

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 9/00, 48/00, A61M 29/00	A1	(11) International Publication Number: WO 97/16169 (43) International Publication Date: 9 May 1997 (09.05.97)
<p>(21) International Application Number: PCT/US96/17311</p> <p>(22) International Filing Date: 30 October 1996 (30.10.96)</p> <p>(30) Priority Data: 60/007,158 1 November 1995 (01.11.95) US 08/726,346 28 October 1996 (28.10.96) US</p> <p>(71) Applicant: CHIRON CORPORATION [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US).</p> <p>(72) Inventor: HUNG, David, T.; 460 Yerba Buena Avenue, San Francisco, CA 94127 (US).</p> <p>(74) Agents: GREEN, Grant, D. et al.; Chiron Corporation, Intellectual Property - R440, P.O. Box 8097, Emeryville, CA 94662-8097 (US).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published</p> <p><i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: TREATMENT OF A CARDIOVASCULAR INDICATION BY DELIVERY OF THERAPEUTICS TO THE PERICARDIAL SPACE</p> <p>(57) Abstract</p> <p>The invention is a treatment for cardiovascular indications involving delivering a polynucleotide therapeutic to the pericardial space for expression therein. Additionally cells can be removed from the pericardial space, transfected with a polynucleotide, and replaced to the pericardial space for expression of the polynucleotide therein. Additionally a drug can be delivered to the pericardial space in combination with the administration of the polynucleotide for increasing the efficacy of treatment of the cardiovascular condition.</p>		

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

TREATMENT OF A CARDIOVASCULAR INDICATION BY DELIVERY OF THERAPEUTICS TO THE PERICARDIAL SPACE

Field of the Invention

5 The invention relates to the delivery of therapeutic agents to the pericardial space for the treatment of a variety of cardiovascular indications. The therapeutic agents of the invention include drugs and polynucleotides; the drugs and polynucleotide therapeutic agents may be formulated in a variety of buffers, excipients, liposomes, particles, polymers, gels, matrices, or any composition
10 facilitating effective delivery to the pericardium, myocardium, coronary vessels, or designated cardiac target location. The polynucleotide therapeutics of the invention may also be incorporated into viral or non-viral vectors for delivery. The therapeutic compositions of the invention are delivered to the pericardial space by a variety of delivery means.

15

Background of the Invention

 Presently, most cardiovascular therapeutics are delivered either orally or intravascularly, by percutaneous cannulation of the systemic venous systems, i.e. standard intravenous therapy. With specialized catheters and delivery devices,
20 cardiovascular therapeutics can be also delivered close to or directly into the heart, through cannulation of the great venous structures (i.e. superior or inferior vena cava, subclavian vein, etc.) or cannulation of the coronary arterial or pulmonary arterial circulation. Because oral or intravascular drug delivery generally results in significant systemic exposure to the agent being delivered,
25 many past methods of drug delivery to the heart have either been limited by complications of systemic toxicity, or have employed suboptimal amounts of therapeutic agents in order to avoid systemic toxicity. A few of the classes of drugs that have been delivered by the above methods to treat a variety of cardiovascular conditions and have been known to result in systemic toxicity
30 include anti-arrhythmics, calcium channel blockers, beta-blockers, contractility improving agents, afterload reducers, preload reducers, immunosuppressive agents, antibiotics, and anti-inflammatory agents. Specific examples of drugs in

- 2 -

each of the above categories, respectively, include amiodarone, verapamil, propranolol, digitalis, hydralazine, nitroglycerin, cyclosporin, isoniazid, and prednisone.

In the treatment of coronary artery restenosis, following percutaneous
5 transluminal coronary angioplasty, the local delivery of anti-restenosis agents directly into the coronary vasculature, either at the endoluminal surface or within the coronary vessel wall via a variety of catheters and coated stents, has become increasingly popular. The basic premise for such delivery is that it allows higher concentrations and more prolonged administration of these agents to be achieved
10 at the pathological site than is practical or achievable with systemic administration. Local drug delivery thereby offers the potential benefits of increasing therapeutic efficacy while reducing systemic toxicity or, in other words, increasing the "therapeutic index" of the delivered agent. However, because the delivery of therapeutic agents to the endoluminal surface of the
15 coronary vasculature generally subjects these agents to the vigorous blood flow of the coronary artery, the retention of these agents at the desired site is often problematic. Drug-impregnated gels or polymers deposited at the endoluminal surface of coronary vessels can prolong drug retention at the desired site but generally suffer from a relatively limited drug storage capacity and, in addition,
20 pose at least theoretical problems related to the gels or polymers themselves such as fragmentation, distal embolization, and inflammation, among other concerns. Prolonged retention of therapeutic agents deposited within the coronary vessel wall itself is hampered by many obstacles including dissipation by drainage by either passive diffusion or via vaso vasorum from the coronary vessel wall and
25 also by the limited capacity of the vessel wall as a drug depot site.

It would be advantageous to develop other methods of treatment of patients having cardiovascular conditions that reduce adverse side effects and heighten efficacy.

30 Summary of the Invention

It is therefore, an object of the present invention to provide a method of treatment or prevention of a cardiovascular indication by administering a

- 3 -

therapeutically effective amount of a drug or a polynucleotide, or a combination of the two locally into the pericardial space.

It is a further object of the invention that the drug or polynucleotide is encapsulated in liposomes, including any liposomal composition such as
5 cyclodextrin liposomes, heterovesicular liposomes, DepoFoam® and synthetic membrane vesicles. It is also contemplated by the invention that the drug or polynucleotide is delivered in other formulations commonly known in the art, including buffers, excipients, polymers, gels such as Focalgel®, and other matrices.

10 Where the therapeutic agent is a polynucleotide, it is an object of the invention that the polynucleotide is in a plasmid or a vector such as a viral vector, alone or also in a liposomal composition or other formulation commonly known in the art.

It is a further object of the invention that the therapeutic agent that is
15 administered into the pericardial space, whether a drug or polynucleotide, or combination of the two, be one of the following: an anti-apoptotic agent, a thrombolytic agent, a pro-angiogenic agent, a complement blocker, an inhibitor of reperfusion injury, an anti-arrhythmic agent, a contractility improving agent, a myocyte growth factor, a vasoactive agent, an anti-hypertensive agent, a calcium
20 channel blocker, a beta-blocker, an afterload reducer, a preload reducer, an anti-inflammatory agent, an immunomodulating or immunosuppressive agent, a free radical scavenging agent, an inhibitor of reactive oxygen metabolites, an anti-thrombotic agent, an anti-platelet agent, an anti-integrin agent, an anti-proliferative agent, a pro-apoptotic agent, an anti-angiogenic agent, and
25 antibiotics, including anti-bacterial, anti-viral, anti-fungal, and anti-parasitic agents, and antitumor agents, including chemotherapeutic agents, radiation sensitizers, and radioactive implants, and a biologically active fragment or chimera of one of these molecules.

Employment of the method of the invention has the further object to
30 prevent, treat, or reduce the attendant effects or complications of the following cardiovascular conditions:

- 4 -

1) atherosclerosis, and conditions that predispose to pathological atherosclerotic plaque development in the coronary arteries including:

- lipid/cholesterol deposition,
- macrophage/inflammatory cell recruitment,
- 5 -plaque rupture,
- thrombosis,
- platelet deposition, and
- neointimal proliferation;

2) ischemic syndromes and attendant syndromes, including but not limited to:

- myocardial infarction,
- stable and unstable angina,
- coronary artery restenosis following percutaneous transluminal coronary angioplasty, and
- 15 -reperfusion injury;

3) cardiomyopathies, including but not limited to cardiomyopathies caused by or associated with:

- ischemic syndromes,
- cardiotoxins such as alcohol and chemotherapeutic agents like
- 20 adriamycin,
- infections, such as viral (CMV) and parasitic, such as caused by *Trypanosoma cruzi*,
- hypertension,
- metabolic diseases, including but not limited to uremia, beriberi,
- 25 glycogen storage disease,
- radiation,
- neuromuscular disease, such as Duchenne's muscular dystrophy,
- infiltrative diseases (including but not limited to sarcoidosis, hemochromatosis, amyloidosis, Fabry's disease, Hurler's
- 30 syndrome,
- trauma, and
- idiopathic causes;

- 4) a/dysrhythmias, including but not limited to a/dysrhythmias resulting from the same causes listed above for cardiomyopathies;
- 5) infections, including bacterial, viral, fungal, and parasitic causes;
- 6) cardiac tumors;
- 5 7) inflammatory conditions, including but not limited to myocarditis, pericarditis, endocarditis, immune cardiac rejection and conditions resulting from idiopathic, autoimmune, or connective tissue diseases; and
- 8) hypertension.

It is a further object of the invention that where the agent used for
10 treatment of coronary artery occlusion, the agent is selected from an inhibitor of lipid or cholesterol synthesis or deposition, such as fish oil, HMG, an inhibitor of macrophage or inflammatory cell recruitment or activation, such as NF- κ B inhibitors like I κ B, pyrrolidine dithiocarbamate (PDTC), and N-acetyl cysteine (NAC), microtubule inhibitors like colchicine and Taxol, anti-inflammatory
15 agents such as those mentioned below, an anti-thrombotic agent as described below under treatment of ischemic syndromes, an antiplatelet agent as described below under treatment of ischemic syndromes, and an inhibitor of neointimal proliferation as described below under anti-proliferative agents.

It is yet another object of the invention that, where the agent used is
20 directed to the prevention, treatment, or reduction of attendant effects of the myocardial ischemic syndromes, the agent is selected from an anti-apoptotic agent, such as an inhibitor of interleukin 1 β converting enzyme; a thrombolytic agent, such as tissue plasminogen activator (TPA), urokinase plasminogen activator (UPA), urokinase, streptokinase, inhibitors of α 2 plasmin inhibitor, and
25 inhibitors of plasminogen activator inhibitor-1; a pro-angiogenic agent, such as basic and acidic fibroblast growth factor, FGF-5, vascular endothelial growth factor, angiogenin, transforming growth factor alpha and beta, tumor necrosis factor alpha, platelet derived growth factor, placental growth factor, hepatocyte growth factor, and proliferin; a complement blocker, such as decay accelerating
30 factor; an inhibitor of reperfusion injury, such as CAB-2; a calcium channel blocker, such as diltiazem; a beta-blocker, such as propranolol; an afterload reducer, such as hydralazine; a preload reducer, such as nitroglycerin; an anti-

thrombotic agent, such as tissue factor pathway inhibitor (TFPI), heparin, hirudin, protein C, protein S, anti-thrombin III, tick anti-coagulant peptide (TAP), and antistasin; an antiplatelet agent, such as a glycoprotein IIb/IIIa antagonist, cyclooxygenase inhibitors like aspirin or non-steroidal anti-inflammatory agents, prostacyclin, or agents that increase platelet cAMP; anti-proliferative agents, such as a ribozymes, antisense oligonucleotides, antibodies, protein, peptide, or small molecule inhibitors against c-myc, ras/raf, PI3 kinase, cyclins, or such as suicide proteins/genes like Herpes thymidine kinase or proapoptotic proteins/genes like fas, faf, interleukin 1b converting enzyme; inhibitor of reactive oxygen metabolites, such as superoxide dismutase, N-acetyl cysteine, pyrrolidine dithiocarbamate (PDMC), vitamin E derivatives, and metal ion chelators; or an anti-angiogenic agent, such as platelet factor 4, thrombospondin, a tissue inhibitor of a metalloproteinase, prolactin, bFGF soluble receptor, angiostatin, TGF- β , interferon- α , and proliferin-related protein. In all cases above where the agent is a protein, a biologically active fragment or a chimera thereof is also contemplated, as is the expression of the gene encoding the protein.

It is a further object of the invention that where the agent used for treatment is intended to improve or prevent cardiomyopathy, the agent is selected from the same list as for treatment of ischemic syndromes above, since ischemic syndromes are a leading cause of cardiomyopathy. In addition, other agents which may be used to treat or prevent cardiomyopathy from other causes include a contractility improving agent, such as digitalis; a myocyte growth factor, such as insulin-like growth factor I (IGF-I), cardioprotective agents, such as Cardioxane; an iron-chelating agent, such as desferoxamine; anti-viral or anti-parasitic agents, a free radical scavenger, such as superoxide dismutase; or the replacement of genes or proteins that may be deficient or downregulated during the development of cardiomyopathy, such as troponin C or the beta-adrenergic receptor. In all cases above where the agent is a protein, a biologically active fragment or a chimera thereof is also contemplated, as is the expression of the gene encoding the protein.

Yet another object of the invention is that where the agent used for treatment is an anti-arrhythmic agent, the agent is selected from any of numerous known anti-

arrhythmics including adenosine, quinidine, propranolol, digoxin, lidocaine, bretylium, amiodarone, and verapamil.

Still another object of the invention is that, where the agent used for treatment is an antibiotic agent, the agent is selected from antibacterial, antivirals anti-fungal, and antiparasitic agents.

Yet another object of the invention is that, where the agent used for treatment is an antitumor agent, the agent is selected from chemotherapeutic agents or radiation sensitizers or radioactive implants.

A further object of the invention is that, where the agent used for treatment is an anti-inflammatory agent, the agent is selected from anti-inflammatory or immunomodulating agents including, but not limited to, steroids, non-steroidal anti-inflammatory agents, cyclosporin, chemotherapeutic agents, and complement inhibitors.

Still another object of the invention is that, where the agent used for treatment is an anti-hypertensive agent, the agent is selected from any of numerous known anti-hypertensive agents including but not limited to hydralazine, propranolol, atrial natriuretic peptide, and endothelin antagonists.

Accordingly, it is an object of the present invention to provide drugs or polynucleotides delivered to the pericardial space, in an appropriate formulation, selected from the group of an anti-apoptotic agent, a thrombolytic agent, a pro-angiogenic agent, an anti-arrhythmic agent, a contractility improving agent, a complement blocker, an inhibitor of reperfusion injury, an calcium channel blocker, a beta-blocker, an afterload reducer, a preload reducer, an antithrombotic agent, an anti-platelet agent, anti-proliferative agent, an anti-inflammatory agent, an immunomodulating agent, an immunosuppressive agent, an inhibitor of reactive oxygen metabolites, an anti-angiogenic agent, a myocyte growth factor, a vasoactive agent, a cardioprotective agent, an iron-chelating agent, an anti-hypertensive agent, an anti-integrin agent, a pro-apoptotic agent, an anti-viral agent, an anti-parasitic agent, a free radical scavenger, and genes or proteins that may be deficient or downregulated during the development of cardiomyopathy, including troponin C or the beta-adrenergic receptor, and a biologically active fragment or a chimera thereof.

Yet another object of the invention is to deliver the therapeutic agent into the pericardial space by injection, catheterization, laser-created perfusion channels, cannulation, particle gun, or pump.

An additional object of the invention is that the access of the therapeutics
5 to the myocardial tissue is increased by such steps including, for example, increasing agent penetration of the myocardium and by creating myocardial perfusion channels by laser. It is an object of the invention that increasing penetration of the myocardium is accomplished by first administering to the pericardial space proangiogenic factors to increase vascularization of myocardial
10 tissue and subsequently administering a therapeutic agent into the pericardial space or specifically formulating agents to increase their tissue penetration.

In accordance with another object of the invention, there is provided a method of augmenting the efficacy of myocardial revascularization by laser by administering a therapeutic agent, such as a proangiogenic agent like bFGF, into
15 the pericardial space in close proximity to the laser revascularization procedure.

In accordance to one of the objects of the present invention, there is provided a method of treatment or prevention of a cardiovascular indication in a patient comprising administering intrapericardially to the patient a pharmaceutical composition comprising a therapeutically effective amount of a
20 first therapeutic agent, wherein the first therapeutic agent comprises a polynucleotide.

In accordance with another object of the present invention, there is provided a kit for intrapericardial delivery of a pharmaceutical composition for treatment or prevention of a cardiovascular indication comprising a catheter and
25 the pharmaceutical composition that comprises a polynucleotide and a drug.

In accordance to a further object of the present invention, there is provided a pharmaceutical composition for intrapericardial delivery for treatment of a cardiovascular indications comprising a first therapeutic agent that comprises a polynucleotide and a drug.

30 In accordance to another object of the invention the polynucleotide is flanked by nucleotide sequence suitable for integration into genome of myocytes.

In accordance to a further object the polynucleotide administered encodes L-aromatic amino acid decarboxylase. In accordance with another object a second therapeutic agent can be administered including L-Dopa, tyrosine, a polynucleotide encoding tyrosine hydroxylase, or a tyrosine hydroxylase polypeptide.

Further objects, features, and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description, while indicating preferred embodiments of the present invention, is given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description. The invention is also not limited to any theories of action of the elements of the invention.

Detailed Description

The invention described herein draws on previously published work and pending patent applications. By way of example, such work consists of scientific papers, patents or pending patent applications. All published work, including patents, and patent applications cited herein are hereby incorporated by reference.

Definitions

The term "cardiovascular indication" as used herein refers to a diagnosis or presumptive diagnosis of cardiovascular disease or conditions affecting the heart that are associated with atherosclerosis, ischemic syndromes, cardiomyopathies, antiyhythmias, dyserhythmias, hypertension and infections. The diagnosis can be made based on pain, fatigability, weakness, palpitations, and systemic symptoms that may be due to the cardiac disease or that may accompany it. Determination of a cardiovascular indication may include a physical exam and other non-invasive diagnostic procedures including radionuclide imaging, positron emission tomography, magnetic resonance imaging, echocardiography, and can also include venous and arterial cannulation and pulmonary and cardiac catheterization used in diagnosis of the cardiac condition.

The term "treatment" as used herein refers to reducing or alleviating symptoms in a subject, preventing symptoms from worsening or progressing, inhibition or elimination of the causative agent, or prevention of the infection or disorder in a subject who is free therefrom. Thus, for example, treatment of a cardiovascular condition in a patient may be reduction of symptoms of heart failure or disfunction, such as reduction of angina, irregular heart beat, or other symptoms of cardiovascular impairment.

The term "prevention" refers to a prophylactic administration in a patient in whom it is expected a cardiovascular condition may develop. A preventative administration might occur before, for example surgery, or might be appropriate where a person has suffered a mild heart attack, and it is feared that a more severe heart attack is likely without some palliative or preventative treatment. A preventative treatment would aim to prevent a harm from occurring to the heart muscle and surrounding tissue, and persons to whom such an administration is most appropriate are persons who are likely candidates for such harm based on other contributing factors or symptoms.

The term "administering intrapericardially" or "administering into the pericardial space" as used herein refers to any method of administration that effects delivery of a therapeutic agent into the pericardial space. The pericardial space may be the entire region comprising the pericardial space, or only a part of it. The term "administering into the pericardial space" is synonymous with the terms "intrapericardial delivery" and "pericardial delivery", and can include delivery to subregions of the pericardial space that form interfaces between the pericardial space and the tissue that surrounds and forms it. The administration into the pericardial space can be accomplished by, for example, the following means of administration including injection, laser, catheter, pump. Intrapericardial delivery of the polynucleotides and the drugs of the invention can be accomplished by the methods of such delivery as disclosed in, for example, U.S. Patent Nos. 5,137,510, 5,269,326, and 5,213,570. herein incorporated by reference.

The term "therapeutically effective amount" as used herein refers to an amount of a therapeutic agent to treat or prevent a cardiovascular condition sufficient to exhibit a

detectable therapeutic or preventative effect. The effect may include, for example, treatment or prevention of the cardiovascular conditions listed herein. The precise effective amount for a subject will depend upon the subject's size and health, the nature and extent of the cardiovascular condition, and the therapeutics or combination of therapeutics selected for administration. Thus, it is not useful to specify an exact effective amount in advance. However, the effective amount for a given situation can be determined by routine experimentation.

The term "therapeutic agent" as used herein encompasses prophylactic agents and refers to any drugs, genes, proteins, polypeptides, peptides and peptoids, or other small molecules and in general, any molecular agent that may be useful to treat or prevent a cardiovascular condition. A therapeutic agent is selected based on the diagnosis of the patient and the particular goals of the therapy. Therapeutic agents for treatment of cardiovascular conditions are numerous and well known, and include but are not limited to the therapeutic agents listed herein. Any therapeutic agent of the invention may be administered with a pharmaceutically acceptable carrier.

The term "pharmaceutically acceptable carrier" refers to a carrier for administration of a therapeutic agent, such as antibodies or a polypeptide, drugs as listed herein, genes, and other therapeutic agents listed herein, *in vivo*, and refers to any pharmaceutical carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which may be administered without undue toxicity. Suitable carriers may be large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Pharmaceutically acceptable salts can be used therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991). Pharmaceutically acceptable carriers in therapeutic compositions may contain liquids such as water, saline, glycerol and ethanol. Additionally,

auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. Typically, the therapeutic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. Liposomes are included within the definition of a pharmaceutically acceptable carrier.

The term "liposomes" refers to, for example, the liposome compositions described in U.S. Patent No. 5,422,120, WO 95/13796, WO 94/23697, WO 91/14445 and EP 524,968 B1. Liposomes may be pharmaceutical carriers for the drugs or polynucleotides or the combination of the two.

The term "drug" as used herein refers to any number of therapeutic molecules, many of which are disclosed herein, that are used for treatment or prevention of cardiovascular conditions. A drug can be a small organic molecule, a peptide, a peptoid, or a polypeptide, for example. Drugs useful herein can include an anti-apoptotic agent, a thrombolytic agent, a pro-angiogenic agent, an anti-arrhythmic agent, a contractility improving agent, a complement blocker, an inhibitor of reperfusion injury, a calcium channel blocker, a beta-blocker, an afterload reducer, a preload reducer, an anti-thrombotic agent, an anti-platelet agent, anti-proliferative agent, an anti-inflammatory agent, an immunomodulating agent, an immunosuppressive agent, an inhibitor of reactive oxygen metabolites, an anti-angiogenic agent, a myocyte growth factor, a vasoactive agent, a cardioprotective agent, an iron-chelating agent, an anti-hypertensive agent, an anti-integrin agent, a pro-apoptotic agent, an anti-viral agent, an anti-parasitic agent, a free radical scavenger, and a protein that may be deficient or downregulated during development of cardiomyopathy, a biologically active fragment thereof and a chimera thereof.

The term "matrices" refers to compositions containing polymers, natural or synthetic, including gels, collagens, gelatin, and fibrinogen.

The term "polynucleotide" as used herein refers to a nucleic acid molecule or a coding sequence that encodes a specific amino acid sequence or its complementary strand. The polynucleotide, nucleic acid molecule or coding sequence can be either DNA or RNA. The DNA molecule can be genomic DNA.

- 13 -

or cDNA. The nucleic acid molecule can be naturally occurring or synthetically made. The nucleic acid molecule can be under control of a regulatory sequence.

A "regulatory sequence" refers to a nucleic acid sequence encoding one or more elements that are capable of affecting or effecting expression of a gene sequence, including transcription or translation thereof, when the gene sequence is placed in such a position as to subject it to the control thereof. Such a regulatory sequence can be, for example, a minimal promoter sequence, a complete promoter sequence, an enhancer sequence, an upstream activation sequence ("UAS"), an operator sequence, a downstream termination sequence, a polyadenylation sequence, an optimal 5' leader sequence to optimize initiation of translation, and a Shine-Dalgarno sequence. Alternatively, the regulatory sequence can contain a combination enhancer/promoter element. The regulatory sequence that is appropriate for expression differs depending upon the host system used for expression. Selection of the appropriate regulatory sequences for use herein is within the capability of one skilled in the art. For example, in prokaryotes, such a regulatory sequence can include one or more of a promoter sequence, a ribosomal binding site, and a transcription termination sequence. In eukaryotes, for example, such a sequence can include one or more of a promoter sequence and/or a transcription termination sequence. Regulatory sequences suitable for use herein may be derived from any source including a prokaryotic source, an eukaryotic source, a virus, a viral vector, a bacteriophage or a linear or circular plasmid. Alternatively, the regulatory sequence herein can be a synthetic sequence such as, for example, one made by combining the UAS of one gene with the remainder of a requisite promoter from another gene, such as the GADP/ADH2 hybrid promoter.

The term "combination" as used herein refers to a combination or mixture of at least one polynucleotide and at least one drug in the same or separate pharmaceutical compositions for administration to the pericardial space according to the invention. A combination is warranted for those cardiovascular indications that respond to both the drug and the polynucleotide when administered together to effect treatment of the cardiovascular condition. The administration of the combination may be simultaneous, or consecutive, in close proximity in time

relative to the responsiveness of each therapeutic. Thus, where a polynucleotide is transformed into the cells of the pericardial tissue within a week of administration into the pericardial space, administration into the pericardial space of a companion drug may occur, for example, up to one week after the initial administration of the polynucleotide and still be considered to have been administered as a combination.

The term "biologically active fragment" refers to fragments of protein or polypeptides that retain one or more of the activities of the full-length protein. In referenced to a protein or polypeptide in the context of the therapeutics and preventatives of the invention and the gene products produced by gene therapy according to the invention, it is contemplated that such protein or polypeptides include biologically active fragments, truncations, variants, alleles, analogs and derivatives thereof. Unless specifically mentioned otherwise, such truncations, variants, alleles, analogs, and derivatives possess one or more of the bioactivities of the native mature protein. This term is not limited to a specific length of the product of the gene. Thus, polypeptides that are identical or contain at least 60%, preferably 70%, more preferably 80%, and most preferably 90% homology to the native protein fragment or the native mature protein, wherever derived, from human or nonhuman sources are included within the term polypeptide. The term polypeptide also does not exclude post-expression modifications of the polypeptide, for example, glycosylations, acrylations, phosphorylations and the like.

"Alleles" and "variants" refers to a polypeptide that differs from the native specified protein by virtue of one or more amino acid substitutions, deletions, or insertions. The amino acid substitutions can be conservative amino acid substitutions or substitutions to eliminate non-essential amino acid residues such as to alter a glycosylation site, a phosphorylation site, an acrylation site, or to alter the folding pattern by altering the position of the cysteine residue that is not necessary for function, etc. Conservative amino acid substitutions are those that preserve the general charge, hydrophobicity/hydrophilicity and/or steric bulk of the amino acid substituted, for example, substitutions between the members of

the following groups are conservative substitutions: Gly/Ala, Val/Ile/Leu, Asp/Glu, Lys/Arg, Asn/Gln, Ser/Cys/Met and Phe/Trp/Tyr.

“Analog” include peptides having one or more peptide mimics, also known as peptoids, that possess the activity sought. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid, including, for example, unnatural amino acids, etc., polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

The term “chimera” as used herein in reference to polynucleotide or a polypeptide denotes a fusion molecule of the coding regions or expression products or portions thereof, respectively, where the coding regions or expression products are not normally contiguous.

A “nucleic acid molecule” or a “polynucleotide,” as used herein, refers to either RNA or DNA molecule that encodes a specific amino acid sequence or its complementary strand. Nucleic acid molecules may also be non-coding sequences, for example, a ribozyme, an antisense oligonucleotide, or an untranslated portion of a gene. A “coding sequence” as used herein, refers to either RNA or DNA that encodes a specific amino acid sequence or its complementary strand. A polynucleotide may include, for example, an antisense oligonucleotide, or a ribozyme, and may also include such items as a 3' or 5' untranslated region of a gene, or an intron of a gene, or other region of a gene that does not make up the coding region of the gene. The DNA or RNA may be single stranded or double stranded. Synthetic nucleic acids or synthetic polynucleotides can be chemically synthesized nucleic acid sequences, and may also be modified with chemical moieties to render the molecule resistant to degradation. Synthetic nucleic acids can be ribozymes or antisense molecules, for example. Modifications to synthetic nucleic acid molecules include nucleic acid monomers or derivative or modifications thereof, including chemical moieties. For example, phosphothioates can be used for the modification. A polynucleotide derivative can include, for example, such polynucleotides as branched DNA (bDNA). A polynucleotide can be a synthetic or recombinant polynucleotide, and can be generated, for example, by polymerase chain reaction

(PCR) amplification, or recombinant expression of complementary DNA or RNA, or by chemical synthesis.

"Coronary artery occlusion" refers to occlusion or reocclusion of a coronary artery as occurs in atherosclerosis, thrombosis, and restenosis.

5 The inventor has discovered that pericardial delivery using gene therapy may be applied to the treatment of cardiovascular conditions since the constitutive or inducible expression of therapeutic gene products in the heart may obviate the need for repeated administration of therapeutic agents or reduce the stringency of requirements for high capacity, prolonged release, long-acting,
10 extremely stable drug formulations. Furthermore, the inducible expression of therapeutic gene products has the advantage of being relatively easily regulatable. Because of the non-proliferative and terminally differentiated state of cardiomyocytes, myocardial somatic gene therapy must employ *in vivo* gene-transfer technologies using vectors and delivery systems that can transduce non-
15 proliferating cells. Previously, delivery of genes to the heart has been accomplished by direct injection of genes encoded in both viral and non-viral vectors to the myocardium. Some of the difficulties with direct myocardial injection include the logistical problems of myocardial trauma and its attendant effects such as myocardial necrosis, fibrosis, inflammation, arrhythmia's, and
20 bleeding problems with delivery of genes to a large myocardial surface area, and the practical limitations of this technique for sustained or repeated administrations.

 Barr *et al.*, *Gene Therapy* (1994) 1:51-58 describes gene delivery via catheter-mediated infusion of replication defective adenovirus into the coronary
25 arterial circulation. High level expression of exogenous gene was obtained throughout the thickness of the ventricular and arterial walls within the distribution of the injected coronary artery. However, infection of multiple cell types and non-cardiac tissues and the binding of adenovirus DNA in the brain and testis raised safety concerns.

30 The inventor herein has discovered that delivery of therapeutic agents including polynucleotides and drugs to the pericardial space results in higher, more prolonged, and more effective drug delivery to arterial structures than local

intra-arterial drug delivery. Deposition of agents within the pericardium is a method of achieving prolonged, high concentration, high capacity drug delivery to the coronary artery.

More prolonged and higher concentration drug delivery is possible from the pericardium as compared to intracoronary delivery because the storage
5 capacity of the pericardium for therapeutic agents is significantly larger than that of the coronary vessel and the agents deposited in the pericardium are not subject directly to flowing blood as are agents deposited directly into coronary artery vasculature. Due to the diffusion of intrapericardially delivered agents across the
10 coronary vessel adventitia into the coronary vascular lumen and, therefore, the coronary artery circulation, deposition within the pericardium is an effective method of achieving prolonged, high concentration, high capacity delivery of therapeutic agents to the myocardium, endocardium, or other designated cardiac targets while minimizing systemic exposure and therefore toxicity.

15 The method of the invention achieves a reduction in systemic toxicity. Systemic toxicity is often experienced with other methods of treating cardiovascular conditions. Because the pericardial sack is a natural closed space the systemic toxicity of agents can be locally contained in this closed space by the method of the invention, and systemic toxicity that is largely influenced and
20 regulatable by the access of the agents to the coronary and myocardial vasculature is controlled.

The invention facilitates access of intrapericardially delivered agents to the myocardium or endocardium by formulations of agents that improve the agents' ability to penetrate deeply into tissues or to penetrate the coronary vessel
25 adventitia, to access the coronary vessel lumen and, therefore, enter the coronary circulation. Also, according to the invention, the creation of either natural or artificial conduits between the pericardial space and the myocardium or endocardium, improves access of intrapericardial agents to these areas of the heart. An example of a method of creating "natural" conduits between the
30 pericardial space and myocardium is the use of proangiogenic factors delivered into the pericardial space to increase the vascularization of the myocardium globally or in a specific area and, therefore, increase the accessibility of agents in

the pericardium to these areas. An example of a method of creating an "artificial" conduit between the pericardial space and myocardium or endocardium is the use of laser, as is currently being used in myocardial revascularization, to create direct channels between these structures to increase the accessibility of agents in the pericardium to deeper myocardial regions.

The inventor has also discovered that delivery of genes to the intrapericardial space is a safer and more effective method of accomplishing myocardial gene therapy. According to the method of the invention, delivery of genes to the pericardial space does not require mechanical violation of the myocardium as does direct myocardial injection. Secondly, because intrapericardially delivered agents have access to the entire myocardial surface the ease and effectiveness with which genes can be delivered to large areas of myocardium is increased. When these agents also, in turn, access the coronary circulation, perfusion of the entire heart with these agents occurs. Third, the inventor has found that the pericardium is more easily transducible than myocardium and, thus, that expression of gene products in the pericardial space retains access to myocardium. The method of administration of the invention is, thus, preferable to previous methods that have attempted expression of gene products in the myocardium with limited success.

The inventor has also found that by the method of the invention the exposure time of nucleic acids and/or viruses to cells, which is an important determinant of transduction or infection efficiency, increases. Genetic agents deposited in the pericardial space are not subject to rapid dilution, drainage, or dissipation due to blood flow or lymphatic clearance, and thus have much longer exposure times than vascularly delivered agents, also increasing the transduction of infection efficiency of the genes. Such an advantage achieved by the method of the invention, translates into much higher transduction or infection efficiency with genes and/or viruses in either the myocardium or the pericardium than is achievable in the coronary vessel. Lastly, because pericardium is highly efficient at expressing certain proteins and in some cases is even more efficient than myocardium at this task, the method of the invention is a new and improved

method of delivery of genes for gene therapy for treatment of a cardiovascular indication.

Practice of the invention also includes, for example, delivering into the pericardial space cardiovascular therapeutics, whether genes or drugs, in liposomal compositions, including heterovesicular liposomes. Delivery in liposomes increases the efficacy of the cardiovascular therapeutic, and reduces the dosage requirements and, in general, augments the benefits of any cardiovascular therapeutic delivered into the pericardial space. Suitable liposomal formats include, for example, the liposome compositions described in U.S. Patent No. 5,422,120, WO 95/13796, WO 94/23697, WO 91/14445 and EP 524,968 B1. Liposomes may be pharmaceutical carriers for the small molecules, polypeptides or polynucleotides of the invention, or for combination of these therapeutics. Liposomes are included within the definition of a pharmaceutically acceptable carrier. Polypeptide therapeutics can also be delivered with the gene that is delivered for expression in the patient, for example, before, with, or after the gene delivery, and polypeptide therapeutic agents can be delivered with any cardiac drugs that are also delivered before, with or after the gene delivery.

The following expression systems detail promoters and vectors useful for gene therapy applications of the invention for the intrapericardial delivery of polynucleotides in plasmids, viral vectors or liposomes, or for the production of proteins that are delivered intrapericardially.

Although the methodology described below is believed to contain sufficient details to enable one skilled in the art to practice the present invention, other items not specifically exemplified, such as plasmids, can be constructed and purified using standard recombinant DNA techniques described in, for example, Sambrook *et al.* (1989), MOLECULAR CLONING: A LABORATORY MANUAL, 2d edition (Cold Spring Harbor Press, Cold Spring Harbor, N.Y.), and Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (1994), (Greene Publishing Associates and John Wiley & Sons, New York, N.Y.), under the current regulations described in United States Dept. of HEW, NATIONAL INSTITUTE OF HEALTH (NIH) GUIDELINES FOR RECOMBINANT DNA RESEARCH. These references include procedures for the following standard methods: cloning procedures with plasmids.

transformation of host cells, cell culture, plasmid DNA purification, phenol extraction of DNA, ethanol precipitation of DNA, agarose gel electrophoresis, purification of DNA fragments from agarose gels, and restriction endonuclease and other DNA-modifying enzyme reactions.

5

Expression in Bacterial Cells

The polynucleotide of the present invention can be produced in prokaryotes, for example bacteria. Further, the proteins and polypeptide drugs of the present invention can also be produced recombinantly in prokaryotes.

- 10 Control elements for use in bacteria include promoters, optionally containing operator sequences, and ribosome binding sites. Useful promoters include sequences derived from sugar metabolizing enzymes, such as galactose, lactose (*lac*) and maltose. Additional examples include promoter sequences derived from biosynthetic enzymes such as tryptophan (*trp*), the β -lactamase (*bla*) promoter
- 15 system, bacteriophage λ PL, and T7. In addition, synthetic promoters can be used, such as the *tac* promoter. The β -lactamase and lactose promoter systems are described in Chang *et al.*, *Nature* (1978) 275: 615, and Goeddel *et al.*, *Nature* (1979) 281: 544; the alkaline phosphatase, tryptophan (*trp*) promoter system are described in Goeddel *et al.*, *Nucleic Acids Res.* (1980) 8: 4057 and EP 36,776 and
- 20 hybrid promoters such as the *tac* promoter is described in U.S. Patent No. 4,551,433 and deBoer *et al.*, *Proc. Natl. Acad. Sci. USA* (1983) 80: 21-25. However, other known bacterial promoters useful for expression of eukaryotic proteins are also suitable. A person skilled in the art would be able to operably ligate such promoters to the coding sequences of interest, for example, as
- 25 described in Siebenlist *et al.*, *Cell* (1980) 20: 269, using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also generally will contain a Shine-Dalgarno (SD) sequence operably linked to the DNA encoding the target polypeptide. For prokaryotic host cells that do not recognize and process the native target polypeptide signal sequence, the signal
- 30 sequence can be substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat

stable enterotoxin II leaders. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria.

The foregoing systems are particularly compatible with *Escherichia coli*. However, numerous other systems for use in bacterial hosts including Gram-negative or Gram-positive organisms such as *Bacillus spp.*, *Streptococcus spp.*, *Streptomyces spp.*, *Pseudomonas* species such as *P. aeruginosa*, *Salmonella typhimurium*, or *Serratia marcescans*, among others. Methods for introducing exogenous DNA into these hosts typically include the use of CaCl_2 or other agents, such as divalent cations and DMSO. DNA can also be introduced into bacterial cells by electroporation, nuclear injection, or protoplast fusion as described generally in Sambrook *et al.* (1989), cited above. These examples are illustrative rather than limiting. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes. Alternatively, *in vitro* methods of cloning, e.g., PCR or other nucleic acid polymerase reactions, are suitable.

Prokaryotic cells used to produce the target polypeptide of this invention are cultured in suitable media, as described generally in Sambrook *et al.*, cited above.

Expression in Yeast Cells

The polynucleotide, proteins and polypeptides and polypeptides of the present invention can also be produced in eukaryotic systems including, for example, yeast cells, insect cells, and mammalian cells. Expression and transformation vectors, either extrachromosomal replicons or integrating vectors, have been developed for transformation into many yeasts. For example, expression vectors have been developed for, among others, the following yeasts: *Saccharomyces cerevisiae*, as described in Hinnen *et al.*, *Proc. Natl. Acad. Sci. USA* (1978) 75: 1929; Ito *et al.*, *J. Bacteriol.* (1983) 153: 163; *Candida albicans* as described in Kurtz *et al.*, *Mol. Cell. Biol.* (1986) 6: 142; *Candida maltosa*, as described in Kunze *et al.*, *J. Basic Microbiol.* (1985) 25: 141; *Hansenula polymorpha*, as described in Gleeson *et al.*, *J. Gen. Microbiol.* (1986) 132: 3459 and Roggenkamp *et al.*, *Mol. Gen. Genet.* (1986) 202 :302; *Kluyveromyces fragilis*, as described in Das *et al.*, *J. Bacteriol.* (1984) 158: 1165; *Kluyveromyces*

- lactis*, as described in De Louvencourt *et al.*, *J. Bacteriol.* (1983) 154: 737 and Van den Berg *et al.*, *BioTechnology* (1990) 8: 135; *Pichia guillermondii*, as described in Kunze *et al.*, *J. Basic Microbiol.* (1985) 25: 141; *Pichia pastoris*, as described in Cregg *et al.*, *Mol. Cell. Biol.* (1985) 5: 3376 and U.S. Patent Nos. 4,837,148 and 4,929,555; *Schizosaccharomyces pombe*, as described in Beach and Nurse, *Nature* (1981) 300: 706; and *Yarrowia lipolytica*, as described in Davidow *et al.*, *Curr. Genet.* (1985) 10: 380 and Gaillardin *et al.*, *Curr. Genet.* (1985) 10: 49; *Aspergillus* hosts such as *A. nidulans*, as described in Ballance *et al.*, *Biochem. Biophys. Res. Commun.* (1983) 112: 284-289; Tilburn *et al.*, *Gene* (1983) 26: 205-221 and Yelton *et al.*, *Proc. Natl. Acad. Sci. USA* (1984) 81: 1470-1474, and *A. niger*, as described in Kelly and Hynes, *EMBO J.* (1985) 4: 475479; *Trichoderma reesia*, as described in EP 244,234, and filamentous fungi such as, e.g., *Neurospora*, *Penicillium*, *Tolypocladium*, as described in WO 91/00357.
- Control sequences for yeast vectors are known and include promoters regions from genes such as alcohol dehydrogenase (ADH), as described in EP 284,044, enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase (GAP or GAPDH), hexokinase, phosphofructokinase, 3-phosphoglycerate mutase, and pyruvate kinase (PyK), as described in EP 329,203. The yeast *PHOS* gene, encoding acid phosphatase, also provides useful promoter sequences, as described in Myanohara *et al.*, *Proc. Natl. Acad. Sci. USA* (1983) 80: 1. Other suitable promoter sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase, as described in Hitzeman *et al.*, *J. Biol. Chem.* (1980) 255: 2073, or other glycolytic enzymes, such as pyruvate decarboxylase, triosephosphate isomerase, and phosphoglucose isomerase, as described in Hess *et al.*, *J. Adv. Enzyme Reg.* (1968) 7:149 and Holland *et al.*, *Biochemistry* (1978) 17:4900. Inducible yeast promoters having the additional advantage of transcription controlled by growth conditions, include from the list above and others the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization.

Suitable vectors and promoters for use in yeast expression are further described in Hitzeman, EP 073,657. Yeast enhancers also are advantageously used with yeast promoters. In addition, synthetic promoters which do not occur in nature also function as yeast promoters. For example, upstream activating sequences (UAS) of one yeast promoter may be joined with the transcription activation region of another yeast promoter, creating a synthetic hybrid promoter. Examples of such hybrid promoters include the ADH regulatory sequence linked to the GAP transcription activation region, as described in U.S. Patent Nos. 4,876,197 and 4,880,734. Other examples of hybrid promoters include promoters which consist of the regulatory sequences of either the *ADH2*, *GAL4*, *GAL10*, or *PHOS* genes, combined with the transcriptional activation region of a glycolytic enzyme gene such as *GAP* or *PyK*, as described in EP 164,556. Furthermore, a yeast promoter can include naturally occurring promoters of non-yeast origin that have the ability to bind yeast RNA polymerase and initiate transcription.

Other control elements which may be included in the yeast expression vectors are terminators, for example, from *GAPDH* and from the enolase gene, as described in Holland *et al.*, *J. Biol. Chem.* (1981) 256: 1385, and leader sequences which encode signal sequences for secretion. DNA encoding suitable signal sequences can be derived from genes for secreted yeast proteins, such as the yeast invertase gene as described in EP 012,873 and JP 62,096,086 and the α -factor gene, as described in U.S. Patent Nos. 4,588,684, 4,546,083 and 4,870,008; EP 324,274; and WO 89/02463. Alternatively, leaders of non-yeast origin, such as an interferon leader, also provide for secretion in yeast, as described in EP 060,057.

Methods of introducing exogenous DNA into yeast hosts are well known in the art and typically include either the transformation of spheroplasts or of intact yeast cells treated with alkali cations.

Transformations into yeast can be carried out according to the method described in Van Solingen *et al.*, *J. Bact.* (1977) 130:946 and Hsiao *et al.*, *Proc. Natl. Acad. Sci. (USA)* (1979) 76:3829. However, other methods for introducing DNA into cells such as by nuclear injection, electroporation, or protoplast fusion may also be used as described generally in Sambrook *et al.*, cited above.

- 24 -

For yeast secretion the native target polypeptide signal sequence may be substituted by yeast signal sequences such as those derived from yeast invertase, α -factor, killer toxins, or acid phosphatase leaders. The origin of replication from the 2 μ plasmid origin is suitable for yeast. A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid described in Kingsman *et al.*, *Gene* (1979) 7: 141 or Tschemper *et al.*, *Gene* (1980) 10:157. The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan. Similarly, Leu2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* Gene.

For intracellular production of the present polypeptides in yeast, a sequence encoding a yeast protein can be linked to a coding sequence of the polypeptide to produce a fusion protein that can be cleaved intracellularly by the yeast cells upon expression. An example, of such a yeast leader sequence is the yeast ubiquitin gene.

Expression in Insect Cells

Baculovirus expression vectors (BEVs) are recombinant insect viruses in which the coding sequence for a foreign gene to be expressed is inserted behind a baculovirus promoter in place of a viral gene, e.g., polyhedrin, as described in Smith and Summers, U.S. Pat. No., 4,745,051.

An expression construct herein includes a DNA vector useful as an intermediate for the infection or transformation of an insect cell system, the vector generally containing DNA coding for a baculovirus transcriptional promoter, optionally but preferably, followed downstream by an insect signal DNA sequence capable of directing secretion of a desired protein, and a site for insertion of the foreign gene encoding the foreign protein, the signal DNA sequence and the foreign gene being placed under the transcriptional control of a baculovirus promoter, the foreign gene herein being the coding sequence of the polypeptide.

The promoter for use herein can be a baculovirus transcriptional promoter region derived from any of the over 500 baculoviruses generally infecting insects, such as, for example, the Orders Lepidoptera, Diptera, Orthoptera, Coleoptera

and Hymenoptera including, for example, but not limited to the viral DNAs of *Autographa californica* MNPV, *Bombyx mori* NPV, *Trichoplusia ni* MNPV, *Rachiplusia ou* MNPV or *Galleria mellonella* MNPV, *Aedes aegypti*, *Drosophila melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia ni*. Thus, the baculovirus transcriptional promoter can be, for example, a baculovirus immediate-early gene IEI or IEN promoter; an immediate-early gene in combination with a baculovirus delayed-early gene promoter region selected from the group consisting of a 39K and a *HindIII* fragment containing a delayed-early gene; or a baculovirus late gene promoter. The immediate-early or delayed-early promoters can be enhanced with transcriptional enhancer elements.

Particularly suitable for use herein is the strong polyhedrin promoter of the baculovirus, which directs a high level of expression of a DNA insert, as described in Friesen *et al.* (1986) "The Regulation of Baculovirus Gene Expression" in: THE MOLECULAR BIOLOGY OF BACULOVIRUSES (W.Doerfler, ed.); EP 127,839 and EP 155,476; and the promoter from the gene encoding the p10 protein, as described in Vlcek *et al.*, *J. Gen. Virol.* (1988) 69:765-776.

The plasmid for use herein usually also contains the polyhedrin polyadenylation signal, as described in Miller *et al.*, *Ann. Rev. Microbiol.* (1988) 42:177 and a procaryotic ampicillin-resistance (*amp*) gene and an origin of replication for selection and propagation in *E. coli*. DNA encoding suitable signal sequences can also be included and is generally derived from genes for secreted insect or baculovirus proteins, such as the baculovirus polyhedrin gene, as described in Carbonell *et al.*, *Gene* (1988) 73:409, as well as mammalian signal sequences such as those derived from genes encoding human α -interferon as described in Maeda *et al.*, *Nature* (1985) 315:592-594; human gastrin-releasing peptide, as described in Lebacqz-Verheyden *et al.*, *Mol. Cell. Biol.* (1988) 8: 3129; human IL-2, as described in Smith *et al.*, *Proc. Natl. Acad. Sci. USA* (1985) 82:8404; mouse IL-3, as described in Miyajima *et al.*, *Gene* (1987) 58:273; and human glucocerebrosidase, as described in Martin *et al.*, *DNA* (1988) 7:99.

Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* host cells have been identified and can be used

herein. See, for example, the description in Luckow *et al.*,

BioTechnology (1988) 6: 47-55, Miller *et al.*, in GENETIC ENGINEERING (Setlow, J.K. *et al.* eds.), Vol. 8 (Plenum Publishing, 1986), pp. 277-279, and Maeda *et al.*, *Nature*, (1985) 315: 592-594. A variety of such viral strains are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV. Such viruses may be used as the virus for transfection of host cells such as *Spodoptera frugiperda* cells.

Other baculovirus genes in addition to the polyhedrin promoter may be employed to advantage in a baculovirus expression system. These include immediate-early (alpha), delayed-early (beta), late (gamma), or very late (delta), according to the phase of the viral infection during which they are expressed. The expression of these genes occurs sequentially, probably as the result of a "cascade" mechanism of transcriptional regulation. Thus, the immediate-early genes are expressed immediately after infection, in the absence of other viral functions, and one or more of the resulting gene products induces transcription of the delayed-early genes. Some delayed-early gene products, in turn, induce transcription of late genes, and finally, the very late genes are expressed under the control of previously expressed gene products from one or more of the earlier classes. One relatively well defined component of this regulatory cascade is IE1, a preferred immediate-early gene of *Autographa californica* nuclear polyhedrosis virus (AcMNPV). IE1 is expressed in the absence of other viral functions and encodes a product that stimulates the transcription of several genes of the delayed-early class, including the preferred 39K gene, as described in Guarino and Summers, *J. Virol.* (1986) 57:563-571 and *J. Virol.* (1987) 61:2091-2099 as well as late genes, as described in Guanno and Summers, *Virol.* (1988) 162:444-451.

Immediate-early genes as described above can be used in combination with a baculovirus gene promoter region of the delayed-early category. Unlike

the immediate-early genes, such delayed-early genes require the presence of other viral genes or gene products such as those of the immediate-early genes. The combination of immediate-early genes can be made with any of several delayed-early gene promoter regions such as 39K or one of the delayed-early gene promoters found on the *Hind*III fragment of the baculovirus genome. In the present instance, the 39 K promoter region can be linked to the foreign gene to be expressed such that expression can be further controlled by the presence of IEI, as described in L. A. Guarino and Summers (1986a), cited above; Guarino & Summers (1986b) *J. Virol.*, (1986) 60:215-223, and Guarino *et al.* (1986c), *J. Virol.* (1986) 60:224-229.

Additionally, when a combination of immediate-early genes with a delayed-early gene promoter region is used, enhancement of the expression of heterologous genes can be realized by the presence of an enhancer sequence in direct cis linkage with the delayed-early gene promoter region. Such enhancer sequences are characterized by their enhancement of delayed-early gene expression in situations where the immediate-early gene or its product is limited. For example, the hr5 enhancer sequence can be linked directly, in cis, to the delayed-early gene promoter region, 39K, thereby enhancing the expression of the cloned heterologous DNA as described in Guarino and Summers (1986a), (1986b), and Guarino *et al.* (1986).

The polyhedrin gene is classified as a very late gene. Therefore, transcription from the polyhedrin promoter requires the previous expression of an unknown, but probably large number of other viral and cellular gene products. Because of this delayed expression of the polyhedrin promoter, state-of-the-art BEVs, such as the exemplary BEV system described by Smith and Summers in, for example, U.S. Pat. No., 4,745,051 will express foreign genes only as a result of gene expression from the rest of the viral genome, and only after the viral infection is well underway. This represents a limitation to the use of existing BEVs. The ability of the host cell to process newly synthesized proteins decreases as the baculovirus infection progresses. Thus, gene expression from the polyhedrin promoter occurs at a time when the host cell's ability to process newly synthesized proteins is potentially diminished for certain proteins such as

- 28 -

human tissue plasminogen activator. As a consequence, the expression of secretory glycoproteins in BEV systems is complicated due to incomplete secretion of the cloned gene product, thereby trapping the cloned gene product within the cell in an incompletely processed form.

5 While it has been recognized that an insect signal sequence can be used to express a foreign protein that can be cleaved to produce a mature protein, the present invention is preferably practiced with a mammalian signal sequence appropriate for the gene expressed.

10 An exemplary insect signal sequence suitable herein is the sequence encoding for a Lepidopteran adipokinetic hormone (AKH) peptide. The AKH family consists of short blocked neuropeptides that regulate energy substrate mobilization and metabolism in insects. In a preferred embodiment, a DNA sequence coding for a Lepidopteran *Manduca sexta* AKH signal peptide can be used. Other insect AKH signal peptides, such as those from the Orthoptera
15 *Schistocerca gregaria* locus can also be employed to advantage. Another exemplary insect signal sequence is the sequence coding for *Drosophila* cuticle proteins such as CP1, CP2, CP3 or CP4.

20 Currently, the most commonly used transfer vector that can be used herein for introducing foreign genes into AcNPV is pAc373. Many other vectors, known to those of skill in the art, can also be used herein. Materials and methods for baculovirus/insect cell expression systems are commercially available in a kit form from companies such as Invitrogen (San Diego CA) ("MaxBac" kit). The techniques utilized herein are generally known to those skilled in the art and are fully described in Summers and Smith, A MANUAL OF METHODS FOR
25 BACULOVIRUS VECTORS AND INSECT CELL CULTURE PROCEDURES, Texas Agricultural Experiment Station Bulletin No. 1555, Texas A&M University (1987); Smith *et al.*, *Mol. Cell. Biol.* (1983) 3: 2156, and Luckow and Summers (1989). These include, for example, the use of pVL985 which alters the polyhedrin start codon from ATG to ATT, and which introduces a *Bam*HI
30 cloning site 32 basepairs downstream from the ATT, as described in Luckow and Summers, *Virology* (1989) 17:31.

Thus, for example, for insect cell expression of the present polypeptides, the desired DNA sequence can be inserted into the transfer vector, using known techniques. An insect cell host can be cotransformed with the transfer vector containing the inserted desired DNA together with the genomic DNA of wild type baculovirus, usually by cotransfection. The vector and viral genome are allowed to recombine resulting in a recombinant virus that can be easily identified and purified. The packaged recombinant virus can be used to infect insect host cells to express a desired polypeptide.

Other methods that are applicable herein are the standard methods of insect cell culture, cotransfection and preparation of plasmids are set forth in Summers and Smith (1987), cited above. This reference also pertains to the standard methods of cloning genes into AcMNPV transfer vectors, plasmid DNA isolation, transferring genes into the AcMNPV genome, viral DNA purification, radiolabeling recombinant proteins and preparation of insect cell culture media. The procedure for the cultivation of viruses and cells are described in Volkman and Summers, *J. Virol.* (1975) 19:820-832 and Volkman *et al.*, *J. Virol.* (1976) 19:820-832.

Expression in Mammalian Cells

Typical promoters for mammalian cell expression of the polypeptides of the invention include the SV40 early promoter, the CMV promoter, the mouse mammary tumor virus LTR promoter, the adenovirus major late promoter (Ad MLP), and the herpes simplex virus promoter, among others. Other non-viral promoters, such as a promoter derived from the murine metallothionein gene, will also find use in mammalian constructs. Mammalian expression may be either constitutive or regulated (inducible), depending on the promoter. Typically, transcription termination and polyadenylation sequences will also be present, located 3' to the translation stop codon. Preferably, a sequence for optimization of initiation of translation, located 5' to the polypeptide coding sequence, is also present. Examples of transcription terminator/polyadenylation signals include those derived from SV40, as described in Sambrook *et al.* (1989), cited

- 30 -

previously. Introns, containing splice donor and acceptor sites, may also be designed into the constructs of the present invention.

Enhancer elements can also be used herein to increase expression levels of the mammalian constructs. Examples include the SV40 early gene enhancer, as described in Dijkema *et al.*, *EMBO J.* (1985) 4:761 and the enhancer/promoter derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus, as described in Gorman *et al.*, *Proc. Natl. Acad. Sci. USA* (1982b) 79:6777 and human cytomegalovirus, as described in Boshart *et al.*, *Cell* (1985) 41:521. A leader sequence can also be present which includes a sequence encoding a signal peptide, to provide for the secretion of the foreign protein in mammalian cells. Preferably, there are processing sites encoded between the leader fragment and the gene of interest such that the leader sequence can be cleaved either *in vivo* or *in vitro*. The adenovirus tripartite leader is an example of a leader sequence that provides for secretion of a foreign protein in mammalian cells.

Once complete, the mammalian expression vectors can be used to transform any of several mammalian cells. Methods for introduction of heterologous polynucleotides into mammalian cells are known in the art and include dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei. General aspects of mammalian cell host system transformations have been described by Axel in U.S. Patent No. 4,399,216.

Gene therapy can be practiced according to the invention by delivery to the pericardial space genes that are under regulatory control of appropriate regulatory sequences for transformation or infection of myocytes, cells within the pericardium, cells at the epicardium, or any cells in a region of the heart accessible to an intrapericardially delivered gene. Gene therapy can be practiced as follows using coding regions for any therapeutic appropriate for treatment of a cardiovascular indication.

The polynucleotide molecule that contain a polypeptide coding sequence for use as a therapeutic agent herein, with or without the coding region for the signal sequence, can be used for treatment of a cardiovascular indication by administration thereof via

gene therapy. Gene therapy strategies for delivery of such constructs can utilize viral or non-viral vector approaches in *in vivo* or *ex vivo* modality. Expression of such coding sequence can be induced using endogenous mammalian or heterologous promoters. Expression of the coding sequence *in vivo* can be either constitutive or regulated.

5 For delivery using viral vectors, any of a number of viral vectors conventional in the art can be used, as described in Jolly, *Cancer Gene Therapy* 1: 51-64 (1994). For example, the UPA coding sequence can be inserted into plasmids designed for expression in retroviral vectors, as described in Kimura *et al.*, *Human Gene Therapy* (1994) 5: 845-852, adenoviral vectors, as described in Connelly *et al.*, *Human Gene Therapy* (1995) 6:
10 185-193, adeno-associated viral vectors, as described in Kaplitt *et al.*, *Nature Genetics* (1994) 6: 148-153 and sindbis vectors. Recombinant retroviruses and various uses thereof have been described in numerous references including, for example, Mann *et al.* (*Cell* 33:153, 1983), Cane and Mulligan (*Proc. Nat'l. Acad. Sci. USA* 81:6349, 1984), Miller *et al.*, *Human Gene Therapy* 1:5-14, 1990, U.S. Patent Nos. 4,405,712; 4,861,719;
15 4,980,289 and PCT Application Nos. WO 89/02,468; WO 89/05,349 and WO 90/02,806, all incorporated by reference in full. Briefly, a foreign gene of interest may be incorporated into the retrovirus in place of the normal retroviral RNA. When the retrovirus injects its RNA into a cell, the foreign gene is also introduced into the cell, and may then be integrated into the host's cellular DNA as if it were the retrovirus itself.
20 Expression of this foreign gene within the host results in expression of the foreign protein by the host cell. Briefly, numerous retroviral gene delivery vehicles may be utilized within the context of the present invention, including for example those described in EP 0,415,731; WO 90/07936; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 93/11230; WO 93/10218; Vile and Hart, *Cancer Res.*
25 53:3860-3864, 1993; Vile and Hart, *Cancer Res.* 53:962-967, 1993; Ram *et al.*, *Cancer Res.* 53:83-88, 1993; Takamiya *et al.*, *J. Neurosci. Res.* 33:493-503, 1992; Baba *et al.*, *J. Neurosurg.* 79:729-735, 1993 (U.S. Patent No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO 91/02805). Particularly preferred recombinant retroviruses include those described in WO 91/02805.

30 Retroviral gene delivery vehicles of the present invention may be readily constructed from a wide variety of retroviruses, including for example, B, C, and D type retroviruses as well as spumaviruses and lentiviruses (see RNA Tumor Viruses, Second

Edition, Cold Spring Harbor Laboratory, 1985). Preferred retroviruses for the preparation or construction of retroviral gene delivery vehicles of the present invention include retroviruses selected from the group consisting of Avian Leukosis Virus, Bovine Leukemia Virus, Murine Leukemia Virus, Mink-Cell Focus-Inducing Virus, Murine Sarcoma Virus, Reticuloendotheliosis virus and Rous Sarcoma Virus. Particularly preferred Murine Leukemia Viruses include 4070A and 1504A (Hartley and Rowe, *J. Virol.* 19:19-25, 1976), Abelson (ATCC No. VR-999), Friend (ATCC No. VR-245), Graffi, Gross (ATCC No. VR-590), Kirsten, Harvey Sarcoma Virus and Rauscher (ATCC No. VR-998), and Moloney Murine Leukemia Virus (ATCC No. VR-190). Such retroviruses may be readily obtained from depositories or collections such as the American Type Culture Collection ("ATCC"; Rockville, Maryland), or isolated from known sources using commonly available techniques.

Any of the above retroviruses may be readily utilized in order to assemble or construct retroviral gene delivery vehicles given the disclosure provided herein, and standard recombinant techniques (e.g., Sambrook et al, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989; Kunkle, *PNAS* 82:488, 1985). In addition, within certain embodiments of the invention, portions of the retroviral gene delivery vehicles may be derived from different retroviruses. For example, retrovector LTRs may be derived from a Murine Sarcoma Virus, a tRNA binding site from a Rous Sarcoma Virus, a packaging signal from a Murine Leukemia Virus, and an origin of second strand synthesis from an Avian Leukosis Virus.

Within preferred aspects of the present invention, recombinant retroviruses may be made by introducing a vector construct as discussed above, into a cell (termed a "packaging cell") which contains those elements necessary for production of infectious recombinant retrovirus which are lacking in the vector construct. A wide variety of retrovector constructs may be utilized within the present invention in order to prepare recombinant retroviruses. For example, retrovector constructs can be provided comprising a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis and a 3' LTR, wherein the vector construct lacks gag/pol or env coding sequences. Briefly, Long Terminal Repeats ("LTRs") are subdivided into three elements, designated U5, R and U3. These elements contain a variety of signals which are responsible for the biological activity of a

retrovirus, including for example, promoter and enhancer elements which are located within U3. LTRs may be readily identified in the provirus due to their precise duplication at either end of the genome. As utilized herein, a 5' LTR should be understood to include a 5' promoter element and sufficient LTR sequence to allow reverse transcription and integration of the DNA form of the vector. The 3' LTR should be understood to include a polyadenylation signal, and sufficient LTR sequence to allow reverse transcription and integration of the DNA form of the vector. The tRNA binding site and origin of second strand DNA synthesis are also important for a retrovirus to be biologically active, and may be readily identified by one of skill in the art. For example, retroviral tRNA binds to a tRNA binding site by Watson-Crick base pairing, and is carried with the retrovirus genome into a viral particle. The tRNA is then utilized as a primer for DNA synthesis by reverse transcriptase. The tRNA binding site may be readily identified based upon its location just downstream from the 5' LTR. Similarly, the origin of second strand DNA synthesis is, as its name implies, important for the second strand DNA synthesis of a retrovirus. This region, which is also referred to as the poly-purine tract, is located just upstream of the 3' LTR. In addition to a 5' and 3' LTR, tRNA binding site, and origin of second strand DNA synthesis, certain preferred retrovector constructs which are provided herein also comprise a packaging signal, as well as one or more nucleic acid molecules (e.g., heterologous sequences), each of which is discussed in more detail below.

Packaging cell lines suitable for use with the above-described retrovector constructs may be readily prepared (see U.S. Serial No. 08/240,030, filed May 9, 1994; see also WO 92/05266), and utilized to create producer cell lines (also termed vector cell lines or "VCLs") for the production of recombinant vector particles. Within particularly preferred embodiments of the present invention packaging cell lines are made from human (e.g., HT1080 cells) or mink parent cell lines, thereby allowing production of recombinant retroviruses that are capable of surviving inactivation in human serum.

Promoters that are suitable for use with these vectors are also conventional in the art and include the Moloney retroviral LTR, CMV promoter and the mouse albumin promoter. Replication incompetent free virus can be produced and injected directly into the animal or humans or by transduction of an autologous cell *ex vivo*, followed by

injection *in vivo* as described in Zatloukal *et al.*, *Proc. Natl. Acad. Sci. USA* (1994) 91: 5148-5152.

The coding sequence of, for example bFGF, VEGF, troponin C, β -adrenergic receptor, myocyte growth factor, DAF, CD59, membrane cofactor protein, ANP, dystrophin, SOD, UPA, angiogenin, TGF- α , TGF- β , G-CSF, placental growth factor, IL-8, hepatocyte growth factor, insulin-like growth factor I (IGF I) proliferin or any of the other recombinant therapeutics listed herein, and chimeric and analog molecules thereof, can also be inserted into plasmids for expression of the polypeptide *in vivo* or *ex vivo*. For *in vivo* therapy, the coding sequence can be delivered into the intrapericardial space by direct injection, or into pericardial tissue by delivery such as, for example, those systems described in U.S. Patent Nos. 5,137,510, 5,213,570, and 5,269,326. Promoters suitable for use in this manner include endogenous and heterologous promoters such as those described herein. Any promoter appropriate for the expression of the gene selected for the therapy is contemplated by the method of the invention. The coding sequence can be injected in a formulation comprising a buffer that can stabilize the coding sequence and facilitate transduction thereof into cells and/or provide targeting, as described in Zhu *et al.*, *Science* (1993) 261: 209-211. Expression of such coding sequence can be induced using endogenous mammalian or heterologous promoters. Expression of the coding sequence *in vivo* can be either constitutive or regulated.

The polynucleotide encoding a desired polypeptide or ribozyme or antisense polynucleotide can also be inserted into plasmid for delivery to cells and where the polynucleotide is a coding sequence, for expression of the desired polypeptide *in vivo*. Promoters suitable for use in this manner include endogenous and heterologous promoters such as CMV. Further, a synthetic T7T7/T7 promoter can be constructed in accordance with Chen *et al.* (1994), *Nucleic Acids Res.* 22: 2114-2120, where the T7 polymerase is under the regulatory control of its own promoter and drives the transcription of polynucleotide sequence, which is also placed under the control of a T7 promoter. The polynucleotide can be injected in a formulation that can stabilize the coding sequence and facilitate transduction thereof into cells and/or provide targeting, as described in Zhu *et al.*, *Science* (1993) 261: 209-211.

Expression of the coding sequence of a desired polypeptide or replication of a ribozyme or antisense polynucleotide *in vivo* upon delivery for gene therapy purposes by

either viral or non-viral vectors can be regulated for maximal efficacy and safety by use of regulated gene expression promoters as described in Gossen *et al.*, *Proc. Natl. Acad. Sci. USA* (1992) 89:5547-5551. For example, the polynucleotide transcription and/or translation can be regulated by tetracycline responsive promoters. These promoters can be regulated in a positive or negative fashion by treatment with the regulator molecule.

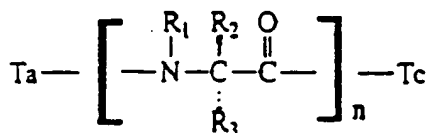
For non-viral delivery of the coding sequence, the sequence can be inserted into conventional vectors that contain conventional control sequences for high level expression, and then be incubated with synthetic gene transfer molecules such as polymeric DNA-binding cations like polylysine, protamine, and albumin, linked to cell targeting ligands such as asialoorosomucoid, as described in Wu and Wu, *J. Biol. Chem.* (1987) 262: 4429-4432; insulin, as described in Hucked *et al.*, *Biochem. Pharmacol.* 40: 253-263 (1990); galactose, as described in Plank *et al.*, *Bioconjugate Chem.* 3:533-539 (1992); lactose, as described in Midoux *et al.*, *Nucleic Acids Res.* 21: 871-878 (1993); or transferrin, as described in Wagner *et al.*, *Proc. Natl. Acad. Sci. USA* 87:3410-3414 (1990). Other delivery systems include the use of liposomes to encapsulate DNA comprising the gene under the control of a variety of tissue-specific or ubiquitously-active promoters, as described in Nabel *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 11307-11311 (1993), and Philip *et al.*, *Mol. Cell Biol.* 14: 2411-2418 (1994). Further non-viral delivery suitable for use includes mechanical delivery systems such as the biolistic approach, as described in Woffendin *et al.*, *Proc. Natl. Acad. Sci. USA* (1994) 91(24): 11581-11585. Moreover, the coding sequence and the product of expression of such can be delivered through deposition of photopolymerized hydrogel materials. Other conventional methods for gene delivery that can be used for delivery of the coding sequence include, for example, use of hand held gene transfer particle gun, as described in U.S. 5,149,655; use of ionizing radiation for activating transferred gene, as described in U.S. 5,206,152 and PCT application WO 92/11033. The aforementioned are not to the exclusion of additional means of facilitating of nucleic acid uptake that rely on nucleic charge neutralization or fusion with cell membranes or facilitate uptake, for example.

Administration a gene for expression in the patient for a non-immunological effect, or a non-coding polynucleotide sequence, can be accomplished by use of a polypeptide, a peptide, a conjugate, a liposome, a lipid, a viral vector, for example, a retroviral vector a non-viral vector.

Polycationic molecules, lipids, liposomes, polyanionic molecules, or polymer conjugates conjugated to the polynucleotide can facilitate non-viral delivery of DNA or RNA. For example, polycationic agents for gene delivery include: polylysine, polyarginine, polyornithine, and protamine. Other examples include histones, protamines, human serum albumin, DNA binding proteins, non-histone chromosomal proteins, coat proteins from DNA viruses, such as ϕ X174, transcriptional factors also contain domains that bind DNA and therefore may be useful as nucleic acid condensing agents, for example, C/CEBP, *c-jun*, *c-fos*, AP-1, AP-2, AP-3, CPF, Prot-1, Sp-1, Oct-1, Oct-2, CREP, and TFIID contain basic domains that bind DNA sequences. Organic polycationic agents include: spermine, spermidine, and putrescine. The dimensions and of the physical properties of a polycationic agent can be extrapolated from the list above, to construct other polypeptide polycationic agents or to produce synthetic polycationic agents.

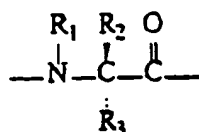
Typically, the polycationic agents exhibit a predicted isoelectric point of at least 9, excluding the terminal groups. The agents contain, excluding the terminal groups, at least 20% basically or positively charged monomers; more typically, at least 25%; more typically, 30%; even more typically, at least 33%; even more typically at least 40%; even more typically, at least 50%; even more typically, at least 60%. Additionally, the agents do not comprises greater than 5% acidic monomers and preferably none. The charge density and composition of the polycationic agent can be altered to accommodate the specific nucleic acid sequence, type, and other components included with the complex of nucleic acids and polycationic agent.

A preferred group of neutral polymers are of the general formula of compounds of the instant invention is as follows:



A preferred subset of these compounds comprise where R_2 is hydrogen. Even more preferred are polymers comprising at least one natural amino acid. Also preferred are polymers where R_2 and R_3 are hydrogen, also referred to as poly N-substituted glycines or poly NSGs.

Monomers will be the general formula as the polycationic monomers with the following structure:



Generally, R₁, R₂, and R₃ are organic moieties each with a molecular weight from 1 to 250 daltons. More typically, the molecular weight is no more than 200; even more typically, no more than 175. Typically, the each monomer comprises one hydrogen at R₁, R₂, or R₃. More, typically, either R₁ and R₃ are both hydrogen, the structure of a L-amino acid; or R₂ and R₃ are both hydrogen, the structure of a NSG. Monomers to be utilized in the neutral agents can be either positively or negatively charged. Also, neutral substituents can also be utilized. The polymers exhibit no net positive or negative charge, excluding the terminal groups.

Degradation sites can be incorporated into the polymers by using naturally occurring amino acid substituents in monomers when R₁ and R₃ are hydrogen. Naturally occurring amino acids and analogues are designated D-amino acids to indicate the chirality of these molecules. L-amino acids can also incorporated as monomers into the neutral polymers. The substituents of L-amino acids can be, for example, the same as those named for the D-amino acids. Also, preferred are NSG to be incorporated as monomers. Preferred monomers are those that capable of forming hydrogen bonds with the polynucleotides to be delivered.

Polymers can be linked together incorporating terminating groups or side chains that permit cross-linking of the polymers. For example, polymers can be linked by a disulfide bond. Other terminating groups useful for coupling polymers include carbonate, urea, and the like. Additional components can be included in the polycationic agents of the instant invention, such as targeting ligands. Such additional groups can facilitate endocytosis of the desired nucleic acids or aid binding of the nucleic acids to the cell surface.

Polypeptides can be incorporated into the polycationic agents. Examples include, without limitation: asialoglycoproteins (ASOR); transferrin; asialoglycoproteins; antibodies; antibody fragments; ferritin; interleukins, interferons, granulocyte

macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), stem cell factor and erythropoietin. Viral antigens, such as envelope proteins, can also be used. Also, proteins from other invasive organisms are useful, such as the 17 amino acid peptide from the circumsporozoite protein of plasmodium falciparum known as RII.

In addition, lipoproteins can be incorporated into the polycationic agent, such as low density lipoprotein, high density lipoprotein, or very low density lipoprotein. Mutants, fragments, or fusions of these proteins can also be used. Other groups that can be incorporated include without limitation: hormones, steroids, androgens, estrogens, thyroid hormone, or vitamins, folic acid. Folic acid can be incorporated into the polycationic agent according, for example, to Mislick, *et al.*, *T.J. Bioconjugate Chem.* 6: 512 (1995). Also, the polycationic agents of the instant invention can be chemically conjugated with polyalkylene glycol. In a preferred embodiment, the polyalkylene glycol is polyethylene glycol. PEG can be incorporated with a polycation agent according, for example, to Lu, *et al.*, *Int. J. Pept. Protein Res.* 43: 127 (1994).

In addition, the polycationic agent can be chemically conjugated with mono-, di-, or polysaccharide. In a preferred embodiment of this aspect, the polysaccharide is dextran. These additional groups can be incorporated within the polycationic agent. For example, R_1 , R_2 , and R_3 can be a substituent that is capable of being activated to cross link with any one of the above groups. For example, a thiol group could be included to cross link with another group to form a disulfide bond.

The terminal groups of the instant polycationic agents can be chosen as convenient. For example, to enhance the targeting properties of the polycationic agent, any of the additional groups described above can be incorporated as terminal groups.

The additional groups described above can be incorporated at the terminus of the polycationic agent. For example, the polycationic agent can be (1) acylated with a variety of carboxylic acids; (2) sulfonylated with sulfonyl chlorides; or (3) derivatized with isocyanates or isothiocyanates. Once activated, the terminus can be reacted with any of the above-mentioned groups, such as a polypeptide, such as low density lipoprotein, or folic acid.

One means of adding a terminal group to the polycationic agent is, for example,

- (1) to acylate the amino terminus with Fmoc-amino-hexanoic acid; and
- (2) to remove the protecting group, Fmoc, to generate a primary amine, which can be further functionalized. Alternatively, the amino-terminal groups can include, without limitation: acyl, such as acetyl, benzoyl; or sulfonyl, such as dansyl. Carboxy terminal
5 groups can include, for example, amide or alkyl amide.

The following is a solid phase method for the synthesis of NSGs, which can be generally used for a wide variety of side-chain substituents. This method can be performed utilizing automated peptide synthesis instrumentation to permit rapid synthesis of polycationic agents of interest. Such instruments are commercially available
10 from, for example, Applied Biosystems and Milligen.

A method of synthesis is to assemble the monomer from two submonomers in the course of extending a polymer comprising a NSG monomer. This technique is described in Zuckermann *et al.*, J Amer Chem Soc 114(26): 10646-10647 (1992) and Zuckermann
15 *et al.*, PCT WO94/06451. The NSGs can also be considered to be an alternating condensation of copolymer of an acylating agent and an amine.

The direction of polymer synthesis with the submonomers occurs in the carboxy to amino direction. The solid-phase assembly for each monomer, in the course of polymer formation, eliminates the need for N α -protected monomers, as only reactive side-chain functionalities need to be protected. Each monomer addition comprises two
20 steps, an acylation step and a nucleophilic displacement step: (1) acylation of a secondary amine bound to the support with an acylating agent comprising a leaving group capable of nucleophilic displacement by an amine and a carbonyl group, preferably carboxyl. An example is a haloacetic acid; and (2) nucleophilic displacement of the leaving group with a sufficient amount of a submonomer comprising a primary
25 amino group to introduce a side-chain. The amino group containing submonomer can be an alkoxyamine, semicarbazide, acyl hydrazide, substituted hydrazine or the like.

Acylation can be activated with carbodimide or other suitable carboxylate activation method. The efficiency of the displacement is modulated by the choice of halide, e.g., I>Cl. Protection of aliphatic hydroxyl groups, carboxylic acids, carboxy,
30 thiol, amino, some heterocycles, and other reactive side-chain functionalities is preferred to minimize undesired side reactions. However, the mild reactivity of some side-chain

moieties toward displacement or acylation may allow their use without protection., e.g., indole, imidazole, and phenol.

NSGs can also be constructed utilizing a three step method for assembling each monomer as the polymer is extended. The backbone of the monomer is first extended by acylation step followed by a nucleophilic displacement. The side chain is introduced by a second acylation step. The backbone of the monomer is assembled in the first two steps of the synthesis cycle. The first reaction is an acylation step where the carbonyl group of the acylating agent reacts with an amine. The acylating agent comprises a carbonyl group; a backbone, R_1 ; and a leaving group, L. Preferably, the carbonyl group is carboxyl.

The second step is a nucleophilic displacement of the leaving group by the first amino group of the displacing agent. The displacing agent comprises a first and a second amino group and a backbone, R_2 . The first amino group is a primary amine, and the second step produces a secondary amine.

The third step is another acylation in which the another acylating submonomer reacts with the first amino group of the displacing agent to produce a tertiary amide. The acylation agent comprises of a carbonyl group; an optional linker; and a sidechain. Preferably, the carbonyl group is carboxyl.

The polycationic agent/polynucleotide complexes, whether or not encapsulated in liposomes, may be administered in pharmaceutical compositions. The pharmaceutical compositions will comprise therapeutically effective amount of nucleic acids. An effective dose for DNA delivery is from about 0.01 mg/kg to 50 mg/kg or 0.05 mg/kg to about 10 mg/kg of the DNA constructs in the individual to which it is administered.

Additional agents can be included with the desired polynucleotides to be delivered, for delivery in either in a gene therapy protocol, or in a nucleic acid vaccination protocol. These additional agents can facilitate, for example, endocytosis of the desired nucleic acids or aid binding of the nucleic acids to the cell surface.

Polypeptides can facilitate DNA delivery and include, for example: asialoglycoproteins; antibodies; antibody fragments; ferritin; interleukins; interferons, granulocyte, macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), stem cell factor and erythropoietin. Viral antigens, such as

envelope proteins, can also be used. Also, proteins from other invasive organisms, such as the 17 amino acid peptide from the circumsporozoite protein of plasmodium falciparum known as RII.

Certain hormones such as for example, steroids, androgens, estrogens, thyroid hormone, or the vitamin, folic acid, can aid in nucleic acid delivery.

Also, polyalkylene glycol can be included with the desired polynucleotides, such as, for example, the polyalkylene glycol is polyethylene glycol. In addition, mono-, di-, or polysaccharides can be included, for example, the polysaccharide dextran or DEAE-dextran, and poly(lactide-co-glycolide)

The desired polynucleotide can also be encapsulated in lipids or packaged in liposomes prior to delivery to the patient. Lipid encapsulation is generally accomplished using liposomes which are able to stably bind or entrap and retain nucleic acid. The ratio of condensed polynucleotide to lipid preparation can vary but will generally be around 1:1 (mg DNA:micromoles lipid), or more of lipid. For a review of the use of liposomes as carriers for delivery of nucleic acids, see, Hug and Sleight, *Biochim. Biophys. Acta* (1991) 1097:1-17; Straubinger *et al.*, in *METHODS OF ENZYMOLOGY* (1983), Vol. 101, pp. 512-527.

Liposomal preparations for use in the instant invention include cationic (positively charged), anionic (negatively charged) and neutral preparations. Cationic liposomes have been shown to mediate intracellular delivery of plasmid DNA (Felgner *et al.*, *Proc. Natl. Acad. Sci. USA* (1987) 84:7413-7416); mRNA (Malone *et al.*, *Proc. Natl. Acad. Sci. USA* (1989) 86:6077-6081); and purified transcription factors (Debs *et al.*, *J. Biol. Chem.* (1990) 265:10189-10192), in functional form.

Cationic liposomes are readily available. For example, N[1-2,3-di(oleoyloxy)propyl]-N,N,N-triethylammonium (DOTMA) liposomes are available under the trademark Lipofectin, from GIBCO BRL, Grand Island, NY. (See, also, Felgner *et al.*, *Proc. Natl. Acad. Sci. USA* (1987) 84:7413-7416). Other commercially available liposomes include transfectace (DDAB/DOPE) and DOTAP/DOPE (Boehringer). Other cationic liposomes can be prepared from readily available materials using techniques well known in the art. See, e.g., Szoka *et al.*, *Proc. Natl. Acad. Sci. USA* (1978) 75:4194-4198; PCT Publication No. WO 90/11092 for a description of the synthesis of DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonio)propane) liposomes.

Similarly, anionic and neutral liposomes are readily available, such as from Avanti Polar Lipids (Birmingham, AL), or can be easily prepared using readily available materials. Such materials include phosphatidyl choline, cholesterol, phosphatidyl ethanolamine, dioleoylphosphatidyl choline (DOPC), dioleoylphosphatidyl glycerol (DOPG), dioleoylphosphatidyl ethanolamine (DOPE), among others. These materials can also be mixed with the DOTMA and DOTAP starting materials in appropriate ratios. Methods for making liposomes using these materials are well known in the art.

The liposomes can comprise multilammellar vesicles (MLVs), small unilamellar vesicles (SUVs), or large unilamellar vesicles (LUVs). The various liposome-nucleic acid complexes are prepared using methods known in the art. See, e.g., Straubinger *et al.*, in METHODS OF ENZYMOLOGY (1983), Vol. 101, pp. 512-527; Szoka *et al.*, *Proc. Natl. Acad. Sci. USA* (1978) 75:4194-4198; Papahadjopoulos *et al.*, *Biochim. Biophys. Acta* (1975) 394:483; Wilson *et al.*, *Cell* (1979) 17:77; Deamer and Bangham, *Biochim. Biophys. Acta* (1976) 443:629; Ostro *et al.*, *Biochem. Biophys. Res. Commun.* (1977) 76:836; Fraley *et al.*, *Proc. Natl. Acad. Sci. USA* (1979) 76:3348; Enoch and Strittmatter, *Proc. Natl. Acad. Sci. USA* (1979) 76:145; Fraley *et al.*, *J. Biol. Chem.* (1980) 255:10431; Szoka and Papahadjopoulos, *Proc. Natl. Acad. Sci. USA* (1978) 75:145; and Schaefer-Ridder *et al.*, *Science* (1982) 215:166.

In addition, lipoproteins can be included with the polynucleotide to be delivered. Examples of lipoproteins to be utilized include: chylomicrons, HDL, IDL, LDL, and VLDL. Mutants, fragments, or fusions of these proteins can also be used. Also, modifications of naturally occurring lipoproteins can be used, such as acetylated LDL. These lipoproteins can target the delivery of polynucleotides to cells expressing lipoprotein receptors. Preferably, if lipoproteins are included with the polynucleotide to be delivered, no other targeting ligand is included in the composition.

If lipoproteins are included with the desired polynucleotides to be delivered, preferably, the composition comprises: lipoprotein, a polynucleotide, and a polynucleotide binding molecule.

Naturally occurring lipoproteins are made up of a lipid and a protein portion. The protein portions of such molecules are known as apoproteins. At the present, apoproteins A, B, C, D, and E have been isolated and identified. At least two of these contain several proteins, designated by Roman numerals, AI, AII, AIV, CI, CII, CIII. A lipoprotein can

comprise more than one apoprotein. For example, naturally occurring chylomicrons comprises of A, B, C, and E, over time these lipoproteins lose A and acquire C and E apoproteins. VLDL comprises A, B, C, and E apoproteins, LDL comprises apoprotein B; and HDL comprises apoproteins A, C, and E. The amino acid of these apoproteins are
5 known and are described in, for example, Breslow, *Ann Rev. Biochem* 54: 699 (1985); Law et al., *Adv. Exp Med. Biol.* 151: 162 (1986); Chen et al., *J Biol Chem* 261: 12918 (1986); Kane et al., *Proc Natl Acad Sci USA* 77: 2465 (1980); and Utermann et al. *Hum Genet* 65: 232: (1984).

Lipoproteins contain a variety of lipids including, triglycerides, cholesterol (free
10 and esters), and phospholipids. The composition of the lipids varies in naturally occurring lipoproteins. For example, chylomicrons comprise mainly triglycerides. A more detailed description of the lipid content of naturally occurring lipoproteins can be found, for example, in Meth. Enzym. 128 (1986). The composition of the lipids are chosen to aid in conformation of the apoprotein for receptor binding activity. The
15 composition of lipids can also be chosen to facilitate hydrophobic interaction and association with the polynucleotide binding molecule.

Naturally occurring lipoproteins can be isolated from serum by ultracentrifugation, for instance. Such methods are described in Meth. Enzy., supra; Pitas et al., *J. Biochem.* 255: 5454-5460 (1980); and Mahey et al., *J Clin. Invest* 64: 743-
20 750 (1979). Lipoproteins can also be produced by in vitro or recombinant methods by expression of the apoprotein genes in a desired host cell. See, for example, Atkinson et al., *Annu Rev Biophys Chem* 15: 403 (1986) and Radding et al., *Biochim Biophys Acta* 30: 443 (1958). Lipoproteins can also be purchased from commercial suppliers, such as Biomedical Technologies, Inc., Stoughton, Massachusetts, USA.

25 Mutants, fragments and fusion of the naturally occurring apoproteins are useful for delivery of polynucleotides. These polypeptides will retain more than about 80% amino acid identity; more typically, more than about 85%; even more typically, at least 90%. Preferably, these polypeptides will exhibit more than about 92% amino acid sequence identity with naturally occurring lipoproteins or fragment thereof; more
30 preferably, more than about 94%; even more preferably, more than about 96%; even more preferably, more than about 98%; even more preferably, more than about 99% sequence identity.

Such mutants, fragments and fusions can be constructed by altering the polynucleotides encoding the desired lipoproteins by recombinant DNA techniques. See, for example, Sambrook et al., (1989) *Molecular Cloning, A Laboratory Manual*, 2d edition (Cold Spring Harbor Press, Cold Spring Harbor, New York). These
5 polynucleotides can be inserted into expression vectors and host cells can be utilized to produce the desired apoprotein.

In addition, naturally occurring lipoproteins, mutants, fragments, and fusions can be chemically altered. For example, acetylated LDL has biological activity. See, for example, Nagelkerke et al., *J. Biol. Chem.* 258(20): 12221-12227 (1983); Weisgraber et al., *J. Biol. Chem.* 253: 9053-9062 (1978); Voyta et al., *J. Cell Biol.* 99: 2034-2040
10 (1984); Goldstein et al., *Proc. Natl. Acad. Sci. USA* 76: 333-337 (1979); and Pitas, *Arteriosclerosis* 1: 177-185 (1981). Chemically modified lipoproteins can also be purchased from commercial suppliers, such as Biomedical Technologies, Inc., Stoughton, Massachusetts, USA.

15 All of these polypeptides exhibit receptor binding properties of naturally occurring lipoproteins. Usually, such polypeptides exhibit at least about 20% receptor binding of naturally occurring lipoproteins. More typically, the polypeptides exhibit at least about 40%, even more typically the polypeptides exhibit at least about 60%; even more typically, at least about 70%; even more typically, at least about 80%; even more
20 typically, at least about 85%; even more typically, at least about 90%; even more typically, at least about 95% receptor binding of the naturally occurring lipoproteins.

Typically, lipoproteins are in an effective amount to increase the frequency of incorporation of polynucleotides into a cell. Such an amount increases the frequency of incorporation of polynucleotides into a cell is at least 10% greater than the frequency of
25 incorporation of naked polynucleotides; more usually, at least 15% greater; even more usually, 20% greater; even more usually, at least 30%. The increase can be between 40 to 100%, and even 1000% and 10000% increase.

A polynucleotide binding molecule refers to those compounds that associate with polynucleotides, and the association is not sequence specific. For example, such
30 molecules can (1) aid in neutralizing the electrical charge of polynucleotide, or (2) facilitate condensation of nucleotides, or (3) inhibit serum or nuclease degradation. Optionally, polynucleotide binding molecules can interact with lipoproteins by either

hydrophobic association or by charge. Polynucleotide binding molecules include, without limitation, polypeptides, mineral compounds, vitamins, etc.

Examples of polynucleotide binding molecules include: polylysine, polyarginine, polyornithine, and protamine. Examples of organic polycations include: spermine, spermidine, and putrescine. Other examples include histones, protamines, human serum albumin. DNA binding proteins, non-histone chromosomal proteins, coat proteins from DNA viruses, such as ϕ X174, transcriptional factors also contain domains that bind DNA and therefore may be useful as nucleic acid condensing agents. Briefly, transcriptional factors such as C/CEBP, c-jun, c-fos, AP-1, AP-2, AP-3, CPF, Prot-1, Sp-1, Oct-1, Oct-2, CREP, and TFIID contain basic domains that bind DNA sequences.

Examples of other positively charged moieties include polybrene, DEAE-dextran, and cationic lipids. Useful cationic lipids and liposomes are described above. Lipids and liposomes are not used in this aspect of the invention to encapsulate both polynucleotide and lipoprotein. The lipoprotein must be exposed to bind the its cell surface receptor.

Other synthetic compounds that are capable of binding negatively charged polynucleotides are useful, such as polymers of N-substituted glycines.

In a composition with a lipoprotein, the polynucleotide binding molecule can be in an amount effective to neutralize the polynucleotide. However, the polynucleotide binding molecule also can be in excess of an effective amount to neutralize the polynucleotide to be delivered. Such an excess can produce a net positive electrical charge when complexed with the polynucleotides to be delivered. The positively charged complex can then interact lipoproteins that comprise negatively charged lipids, such as phospholipids.

Typically, the polynucleotide binding molecule is in excess when the amount is 10% greater than the amount to neutralize the polynucleotide charge; more typically, the amount is 50% greater; even more typically, 100% greater; even more typically, 150% greater; even more typically, 200% greater; even more typically, 500% greater; even more typically, 20,000% greater; even more typically, 22,000% greater; even more typically, 25,000% greater; even more typically, 30,000% greater; even more typically, more than 40,000% greater than the amount effective to neutralize the electrical charge of the desired polynucleotide.

To practice the invention, the diagnosis of a cardiovascular condition is made, and the appropriate therapeutic agent or agents and dosages are determined on the basis of the diagnosis. The invention is practiced to prevent, reduce or treat a cardiovascular condition. Diagnosis can include diagnosis of the cardiovascular condition can be made using standard techniques.

A therapeutic agent can be administered to a patient with a cardiovascular condition, or a patient at risk for developing a cardiovascular condition, in a protocol that includes administration of several therapeutic agents. Any of these therapeutic agents can be incorporated into an appropriate pharmaceutical composition that includes a pharmaceutically acceptable carrier for the agent. Suitable carriers may be large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Pharmaceutically acceptable salts can be used therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991). Pharmaceutically acceptable carriers in therapeutic compositions may contain liquids such as water, saline, glycerol and ethanol. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. Typically, the therapeutic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. Liposomes are included within the definition of a pharmaceutically acceptable carrier. The term "liposomes" refers to, for example, the liposome compositions described in U.S. Patent No. 5,422,120, WO 95/13796, WO 94/23697, WO 91/14445 and EP 524,968 B1. Liposomes may be pharmaceutical carriers for the small molecules, polypeptides or polynucleotides of the invention, or for combination of these therapeutics.

Additionally, administration of the therapeutic agents of the invention can be accomplished for example, in a simultaneous administration, in sequential administration, and with the same or different pharmaceutically acceptable carriers, as is

appropriate for best accomplishing the goal of improving the patient's condition.

Further, a therapeutic composition can be administered that includes all the therapeutic agents necessary to achieve the therapeutic goals of the therapy.

Administration of a therapeutic of the invention, includes administering a
5 therapeutically effective dose of the therapeutic, by a means considered or empirically deduced to be effective for inducing the desired, therapeutic effect in the patient. Both the dose and the administration means can be determined based on the specific qualities of the therapeutic, the condition of the patient, the progression of the disease, and other relevant factors. Administration for the therapeutic agents of the invention can include,
10 for example, any administration targeted to releasing the therapeutic agent in the pericardial space, including for example parenteral administration, including injection, catheterization, laser-created perfusion channels, a particle gun, and a pump. Parenteral administration can be, for example, intravenous, subcutaneous, intradermal, or intramuscular, administration.

15 The therapeutics of the invention can be administered in a therapeutically effective dosage and amount, in the process of a therapeutically effective protocol for treatment of the patient. The initial and any subsequent dosages administered will depend upon the patient's age, weight, condition, and the disease, disorder or biological condition being treated. Depending on the therapeutic, the dosage and protocol for
20 administration will vary, and the dosage will also depend on the method of administration selected, for example, local or systemic administration.

For polypeptide therapeutics, for example, the dosage can be in the range of about 5 μ g to about 50 μ g/kg of patient body weight, also about 50 μ g to about 5 mg/kg, also about 100 μ g to about 500 μ g/kg of patient body weight, and about 200 to about 250
25 ug/kg.

For polynucleotide therapeutics, depending on the expression of the polynucleotide in the patient, for tissue targeted administration, vectors containing expressable constructs of coding sequences, or non-coding sequences can be administered in a range of about 100 ng to about 200 mg of DNA for local administration
30 in a gene therapy protocol, also about 500 ng to about 50 mg, also about 1 ug to about 2 mg of DNA, about 5 ug of DNA to about 500 ug of DNA, and about 20 ug to about 100

ug during a local administration in a gene therapy protocol, and for example, a dosage of about 500 ug, per injection or administration.

Non-coding sequences that act by a catalytic mechanism, for example, catalytically active ribozymes may require lower doses than non-coding sequences that are held to the restrictions of stoichiometry, as in the case of, for example, antisense molecules, although expression limitations of the ribozymes may again raise the dosage requirements of ribozymes being expressed *in vivo* in order that they achieve efficacy in the patient. Factors such as method of action and efficacy of transformation and expression are therefore considerations that will effect the dosage required for ultimate efficacy for DNA and nucleic acids. Where greater expression is desired, over a larger area of tissue, larger amounts of DNA or the same amounts readministered in a successive protocol of administrations, or several administrations to different adjacent or close tissue portions of for example, a tumor site, may be required to effect a positive therapeutic outcome.

For administration of small molecule therapeutics, depending on the potency of the small molecule, the dosage may vary. For a very potent inhibitor, microgram (μ) amounts per kilogram of patient may be sufficient, for example, in the range of about 1 μ g/kg to about 500 mg/kg of patient weight, and about 100 μ g/kg to about 5 mg/kg, and about 1 μ g/kg to about 50 μ g/kg, and, for example, about 10 ug/kg. For administration of peptides and peptoids the potency also affects the dosage, and may be in the range of about 1 μ g/kg to about 500 mg/kg of patient weight, and about 100 μ g/kg to about 5 mg/kg, and about 1 μ g/kg to about 50 μ g/kg, and a usual dose might be about 10 ug/kg.

In all cases, routine experimentation in clinical trials will determine specific ranges for optimal therapeutic effect, for each therapeutic, each administrative protocol, and administration to specific patients will also be adjusted to within effective and safe ranges depending on the patient condition and responsiveness to initial administrations.

The method of the invention applies to any cardiovascular indication, for example a diagnosis of: (1) atherosclerosis, and conditions that predispose one to pathological atherosclerotic plaque development in the coronary arteries including lipid/cholesterol deposition, macrophage/inflammatory cell recruitment, plaque rupture, thrombosis, platelet deposition, neointimal proliferation; (2) ischemic syndromes and attendant

syndromes, including but not limited to myocardial infarction, stable and unstable angina, coronary artery restenosis following percutaneous transluminal, coronary angioplasty, reperfusion injury; (3) cardiomyopathies, including but not limited to cardiomyopathies caused by ischemic syndromes, cardiotoxins such as alcohol and
5 chemotherapeutic agents like adriamycin, infections, such as viral, cytomegalovirus (CMV), and parasitic (*trypanosoma cruzi*), hypertension, metabolic diseases, (including but not limited to uremia, beriberi, glycogen storage disease), radiation, neuromuscular disease (such as Duchenne's muscular dystrophy), infiltrative diseases (including but not limited to sarcoidosis, hemochromatosis, amyloidosis, Fabry's disease, Hurler's
10 syndrome), trauma, and idiopathic causes; (4) a/dysrhythmias (including but not limited to a/dysrhythmias resulting from the same causes listed above for cardiomyopathies); (5) infections (including bacterial, viral, fungal, and parasitic causes); (6) cardiac tumors; (7) inflammatory conditions (including but not limited to myocarditis, pericarditis, endocarditis, immune cardiac rejection and conditions resulting from idiopathic,
15 autoimmune, or connective tissue diseases); and (8) hypertension.

The therapeutic agent selected for practice of the invention can be a drug or a polynucleotide, or a combination of the two, or a combination of more than one drug, or a combination of more than one polynucleotide. Where a drug is selected, the appropriate formulation of the drug for minimizing a detrimental
20 immunological response and for maximizing the effectiveness of the drug, including perfusion of the drug, is also selected. Some appropriate formulations include buffers, excipients, gels, matrices and polymers known in the art of drug delivery. Appropriate formulations for drugs used in the practice of the invention also include liposomal preparations such as, for example, those disclosed in U.S.
25 Patent No. 5,422,120, WO 95/13796, WO 94/23697, WO 91/14445 and EP 0 524 968-B1, particularly including heterovesicular liposomal preparations. Formulations of liposomes provide an increased and sustained delivery of drugs much improved from the prior formulations, and such formulations are particularly well-suited to the method of this invention.

30 Where the therapeutic agent selected for treatment is a polynucleotide that is then administered to the pericardial space and expressed in the heart tissue, including but not limited to, for example, pericardial tissue, myocardial tissue,

epicardial tissue, or perivascular tissue, the polynucleotide is isolated and placed in a vector. The vector is, for example, a viral vector, or a plasmid vector. The regulatory sequences are any regulatory sequence appropriate the polynucleotide and other parameters comprising the gene therapy, and can be any appropriate regulatory sequence or combination of sequences. The polynucleotides may be presented into the pericardial space in any formulation commonly known in the art including buffers, excipients, gels, matrices and polymers. Appropriate formulations for the polynucleotides administered intrapericardially in the practice of the invention also include liposomal preparations such as, for example, those disclosed in U.S. Patent No. 5,422,120, WO 95/13796, WO 94/23697, WO 91/14445 and EP 524,968 B1, particularly including the heterovesicular liposomal preparations disclosed in these patents and applications.

The polynucleotide for delivery into the pericardial space can be prepared by any method conventional in the art, such as that described in Barr *et al.*, *Gene Therapy* (1994) 1:51-58, using replication deficient adenoviral vectors. The polynucleotide for intrapericardial delivery may be linked to tissue specific promoters or leader sequences for expression in cardiac muscle cells, for example, the untranslated leader sequence of dystrophin DNA, or regulatory regions muscle creatine kinase gene such as that described in Cox *et al.*, *Nature* (1993) 364:725-729.

Where the therapeutic agent of the invention is a combination of a drug and a polynucleotide, appropriate formulations containing pharmaceutically acceptable carriers are also prepared for the combination. In addition, the drug and the polynucleotide can be prepared in separate formulations, of the same formulation, and can be administered simultaneously or consecutively.

The drugs and polynucleotides that are appropriate for the method of the invention include any cardiac drug or any polynucleotide that is known to or is expected to prevent, reduce or treat a cardiovascular condition. Some examples of such drugs and polynucleotides are listed below. They include, but are not limited to:

- (1) an agent used for treatment of coronary artery occlusion or reocclusion (as occurs in atherosclerosis, thrombosis, or restenosis), including an inhibitor of lipid/cholesterol synthesis/deposition (such as, for example, fish oil, HMG), and an inhibitor of macrophage/inflammatory cell recruitment or activation, such as, for example, NF- κ B inhibitors like I κ B, pyrrolidine dithiocarbamate, and N-acetyl cysteine, microtubule inhibitors like colchicine and Taxol, and any antiinflammatory agent, any antithrombotic agent, any antiplatelet agent, and an inhibitor of neointimal proliferation;
- (2) an agent directed at the prevention, treatment, or reduction of attendant effects of the myocardial ischemic syndromes, including, for example,
- (a) an anti-apoptotic agent, such as, for example, an inhibitor of interleukin 1b converting enzyme;
- (b) a thrombolytic agent, such as, for example, tissue plasminogen activator (TPA), urokinase plasminogen activator (UPA), urokinase, streptokinase, an inhibitor of α 2 plasmin inhibitor, and an inhibitor of plasminogen activator inhibitor-1;
- (c) a pro-angiogenic agent, such as, for example, basic and acidic fibroblast growth factor, FGF-5, vascular endothelial growth factor, angiogenin, transforming growth factor alpha and beta, tumor necrosis factor alpha, platelet derived growth factor, placental growth factor, hepatocyte growth factor, and proliferin;
- (d) a complement blocker, such as, for example, decay accelerating factor;
- (e) an inhibitor of reperfusion injury, such as, for example, CAB-2;
- (f) a calcium channel blocker, such as, for example, diltiazem;
- (g) a beta-blocker, such as, for example, propranolol;
- (h) an afterload reducer, such as, for example, hydralazine;
- (i) a preload reducer, such as, for example, nitroglycerin;
- (j) an anti-thrombotic agent, such as, for example, tissue factor pathway inhibitor, heparin, hirudin, protein C, protein S, anti-thrombin III, tick anti-coagulant peptide (TAP), and antistasin;

(k) an antiplatelet agent, such as, for example, a glycoprotein IIb/IIIa antagonist;

(l) a cyclooxygenase inhibitor, such as, for example, aspirin or non-steroidal anti-inflammatory agents, prostacylin, or agents that increase platelet cAMP;

(m) an anti-proliferative agent, such as, for example, ribozymes, antisense oligonucleotides, antibodies, protein, peptide, or small molecule inhibitors against c-myc, ras/raf, PI3 kinase, cyclins, or such as, for example, suicide proteins/genes such as, for example, herpes thymidine kinase or proapoptotic proteins/genes like fas, fap, interleukin 1 β converting enzyme;

(n) an inhibitor of reactive oxygen metabolites, such as, for example, superoxide dismutase, N-acetyl cysteine, pyrrolidine dithiocarbamate, vitamin E derivatives, and metal ion chelators; and

(o) an antiangiogenic agent, such as, for example, platelet factor 4, thrombospondin, a tissue inhibitor of a metalloproteinase, prolactin, bFGF soluble receptor, angiostatin, TFG- β , interferon- α , and proliferin-related protein;

(3) an agent to prevent, reduce, or treat cardiomyopathy, including

(a) the above list for ischemic syndromes; and also including
(b) a contractility improving agent, such as, for example, digitalis;

(c) a myocyte growth factor, such as insulin-like growth factor I (IGF-I);

(d) a cardioprotective agent, such as, for example, Cardioxane;
(e) an iron-chelating agent, such as, for example, desferoxamine;

(f) an anti-viral or anti-parasitic agent,

(g) a free radical scavenger, such as, for example, superoxide dismutase; and

(h) the replacement of genes or proteins which may be deficient or downregulated during the development of cardiomyopathy, such as, for example, troponin C or the beta-adrenergic receptor.

(4) an antiarrhythmic agent including, for example, adenosine, quinidine, propranolol, digoxin, lidocaine, bretylium, amiodarone, and verapamil;

(5) an antibiotic agent, including, for example, antibacterial, antivirals anti-fungal, and antiparasitic agents;

5 (6) an anti-tumor agent, including, for example, chemotherapeutic agents or radiation sensitizers or radioactive implants;

(7) an anti-inflammatory agent including, for example, an anti-inflammatory or immunomodulating agent, such as, for example, non-steroidal anti-inflammatory agents, cyclosporin, chemotherapeutic agents, and complement inhibitors; and

10 (8) an anti-hypertensive agent, including but not limited to, for example, hydralazine, propranolol, atrial natriuretic peptide, and endothelin antagonists.

Administration of the therapeutic agent into the pericardial space is accomplished by a means appropriate to the cardiovascular condition of the patient, the therapeutic agent and its formulation, and the goals of the treatment. Repeated or continuous administration over a period of time is also contemplated by the invention. In general, any method appropriate for local delivery of a therapeutic to any part of the body is appropriate for administration into the pericardial space. These methods include, for example, injection, catheterization, laser-created perfusion channels, cannulization, a particle gun, and a pump.

20 In order to maximize the perfusion of the myocardial tissues during the treatment by the method of the invention, the invention can be practiced by first administering to the pericardial space proangiogenic factors to increase vascularization of myocardial tissue, formulating agents such that tissue penetration by the agent is enhanced, or by first creating perfusion channels in the myocardial tissue with a laser. Subsequently, the therapeutic agent is administered into the pericardial space, and the perfusion to tissue and cells of the agent is increased.

Also, a therapeutically effective amount of an anti-inflammatory agent to counteract an inflammatory response to administration of the polynucleotide can be administered either before, contemporaneously with, or after administration of the polynucleotide.

The pharmaceutical agent including a polynucleotide can be a polynucleotide that encodes a polypeptide including for example, anti-apoptotic agent, a thrombolytic agent, a pro-angiogenic agent, an anti-arrhythmic agent, a contractility improving agent, a complement blocker, an inhibitor of reperfusion injury, an calcium channel blocker, a beta-blocker, an afterload reducer, a preload reducer, an anti-thrombotic agent, an anti-platelet agent, anti-proliferative agent, an anti-inflammatory agent, an immunomodulating agent, an immunosuppressive agent, an inhibitor of reactive oxygen metabolites, an anti-angiogenic agent, a vasoactive agent, a cardioprotective agent, an iron-chelating agent, an anti-hypertensive agent, an anti-integrin agent, a pro-apoptotic agent, an anti-viral agent, an anti-parasitic agent, a free radical scavenger, a protein that may be deficient or downregulated during the development of cardiomyopathy, a biologically active fragment thereof, or a chimera thereof.

The drug also part of the pharmaceutical that includes the polynucleotide can include, for example, an anti-apoptotic agent, a thrombolytic agent, a pro-angiogenic agent, an anti-arrhythmic agent, a contractility improving agent, a complement blocker, an inhibitor of reperfusion injury, a calcium channel blocker, a beta-blocker, an afterload reducer, a preload reducer, an anti-thrombotic agent, an anti-platelet agent, anti-proliferative agent, an anti-inflammatory agent, an immunomodulating agent, an immunosuppressive agent, an inhibitor of reactive oxygen metabolites, an anti-angiogenic agent, a myocyte growth factor, a vasoactive agent, a cardioprotective agent, an iron-chelating agent, an anti-hypertensive agent, an anti-integrin agent, a pro-apoptotic agent, an anti-viral agent, an anti-parasitic agent, a free radical scavenger, and a protein that may be deficient or downregulated during development of cardiomyopathy, or a biologically active derivative thereof.

Efficacy of myocardial revascularization by laser can be augmented by administering a therapeutic agent, such as a proangiogenic agent like basis fibroblast growth factor (bFGF), or a gene encoding bFGF, into the pericardial space in close proximity to the laser revascularization procedure.

Treatment of cardiomyopathy can be accomplished by administering to the pericardial space agents that improve contractility of the myocardial tissue. For example, it is known that severe arterial hypotension that is unresponsive to volume replacement can be treated in titrated doses with continuous infusion of dopamine at an amount and

concentration of about 400 mg/250ml of 5% D/W (1.6mg/ml) beginning at 3 to 5 ug/kg/min, and including also epinephrine, norepinephrine or phenylephrine as described in THE MERCK MANUAL, (Berkow Ed., Merck Res. Lab., Rahway, N.J. 16th Edition (1992), pg. 534. In addition, other administrative protocols are possible, for example, 5 administering a polynucleotide encoding L-aromatic amino acid decarboxylase to the pericardial space. L-aromatic amino acid decarboxylase converts L-Dopa to dopamine which drug can improve contractility of myocardial tissue. L-aromatic amino acid decarboxylase can be administered either in the polynucleotide form for expression in cells in the pericardial space, the polynucleotide form transfected into cells removed 10 from the pericardial space which are then readministered to the pericardial space for expression of the of L-aromatic amino acid decarboxylase, or in the polypeptide form of L-aromatic amino acid decarboxylase. L-aromatic amino acid decarboxylase allows the metabolism of L-Dopa to dopamine, an active agent in improving cardiac muscle contractility. Coincident with or in combination with an administration of L-aromatic amino acid decarboxylase, can thus be administered any combination of the following: 15 L-Dopa systemically or L-Dopa locally to the pericardial space. Additionally, for example, the elements which make up L-Dopa can be administered: including tyrosine and the enzyme that converts tyrosine to L-Dopa called tyrosine hydroxylase. Tyrosine hydroxylase can be administered as a protein having activity or as a polynucleotide for 20 expression in the cells of the pericardial space, or some cells of the pericardium can be removed and transfected with the tyrosine hydroxylase and the transfected cells can be returned to the pericardial space for expression of the enzyme therein.

Any polynucleotide for administration to the patient can be expressed *in vitro* (in cells removed from the patient and replaced after transformation into the pericardial space) or *in vivo* (in the patient by administration of the polynucleotide into the 25 pericardial space) from a viral vector, including, for example, a vector derived from a retrovirus, an adenovirus, an adeno-associated virus, a herpes virus, an alpha virus, a semliki forest virus, or a sindbis virus. The viral vector can include a gene, for example a suicide gene, for the purpose of inactivating expression of the polynucleotide at an appropriate or necessary time. Thus the viral vector capable of expressing the 30 polynucleotide therapeutic can also contain, for example, a thymidine kinase gene from the Herpes simplex virus. Gancyclovir can be administered to the patient and a cell

expressing the thymidine kinase will enable phosphorylation of gancyclovir which makes gancyclovir become toxic which kills the cell. Thus, the expression of the polynucleotide of interest is stopped. Other genes which when combined with a pro-drug like gancyclovir can also be used for this purpose, for example the PNP gene.

5 A chaperone molecule can be administered before, contemporaneously with or after administration of the polynucleotide therapeutic, and the chaperone molecule can be, for example, a heat shock protein, such as, for example hsp70. Further, the polynucleotide being expressed in the cardiac patient can be linked to an inducible promoter, for example a heart tissue specific promoter, for the
10 purpose of, for example, for ensuring expression of the polynucleotide only in the myocytes adjacent to the pericardial space. Additionally, for the purpose of effectively delivering the polynucleotide to heart tissue, the polynucleotide can be flanked by nucleotide sequences suitable for integration into genome of myocytes.

15 Further objects, features, and advantages of the present invention will become apparent from the detailed description. It should be understood, however, that the detailed description, while indicating preferred embodiments of the invention, is given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed
20 description. Also, the invention is not limited by any theories of mechanism of the method of the invention.

Example 1

25 A coding sequence for bFGF is isolated by standard recombinant DNA techniques and placed in a plasmid vector having appropriate regulatory regions for expression of the bFGF polypeptide in a myocyte. The plasmid is delivered by laparoscopic cannulation to the pericardial space of a cardiac patient who has suffered a myocardial infarction. The plasmid is delivered as naked DNA, in an
30 amount appropriate for the patient, in a liposomal preparation.. Also administered in the pharmaceutical composition is a heparin polypeptide. The patient is monitored for improved heart function and readministration of the

bFGF polynucleotide with the heparin polypeptide is repeated for several administrations as necessary for patient recovery.

This Page Blank (uspto)

What Is Claimed:

1. A method of treatment or prevention of a cardiovascular indication in a patient comprising,
 - (a) providing a pharmaceutical composition comprising a therapeutically effective amount of a first therapeutic agent, that comprises a polynucleotide, and
 - (b) administering intrapericardially to the patient the pharmaceutical composition.
2. The method of claim 1, the pharmaceutical composition further comprising a therapeutically effective amount of a second therapeutic agent, that comprises a drug.
3. The method of claim 1, comprising administering to the patient a second therapeutic agent, wherein the second therapeutic agent is administered either before, contemporaneously with or after the administration of the first therapeutic agent.
4. The method of claim 1, wherein the polynucleotide is associated with at least one selected from the group consisting of a liposome, cyclodextrin liposome, heterovesicular liposome, a synthetic membrane vesicle, a gel, a polymer, an excipient, matrices, a charged particle and a buffer.
5. The method of claim 4, wherein the liposome is a cyclodextrin liposome or a heterovesicular liposome.
6. The method of claim 1, wherein the polynucleotide comprises a plasmid.
7. The method of claim 1, wherein the polynucleotide comprises a viral vector.

8. The method of claim 7, comprises a viral vector selected from the group consisting of a retrovirus, an adenovirus, an adeno-associated virus, a herpes virus, an alpha virus, a semliki forest virus, and a sindbis virus.

9. The method of claim 2, wherein the drug is associated with at least one selected from liposome, a synthetic membrane vesicle, a gel, a polymer, an excipient, matrices, and a buffer.

10. The method of claim 3, wherein the polynucleotide and the drug are associated with at least one selected from the group consisting of a liposome, a synthetic membrane vesicle, a gel, a polymer, an excipient, matrices, a charged particle and a buffer.

11. The method of claim 2, comprising a second therapeutic agent selected from the group consisting of an anti-apoptotic agent, a thrombolytic agent, a pro-angiogenic agent, an anti-arrhythmic agent, a contractility improving agent, a complement blocker, an inhibitor of reperfusion injury, a calcium channel blocker, a beta-blocker, an afterload reducer, a preload reducer, an anti-thrombotic agent, an anti-platelet agent, anti-proliferative agent, an anti-inflammatory agent, an immunomodulating agent, an immunosuppressive agent, an inhibitor of reactive oxygen metabolites, an anti-angiogenic agent, a myocyte growth factor, a vasoactive agent, a cardioprotective agent, an iron-chelating agent, an anti-hypertensive agent, an anti-integrin agent, a pro-apoptotic agent, an anti-viral agent, an anti-parasitic agent, a free radical scavenger, and a protein that may be deficient or downregulated during development of cardiomyopathy, and a biologically active derivative thereof.

12. The method for claim 1, wherein the polynucleotide encodes a protein that may be deficient or downregulated during development of cardiomyopathy.

13. The method of claim 12, wherein the protein that may be deficient or downregulated during development of cardiomyopathy is troponin C.

14. The method of claim 11, wherein the polynucleotide encodes a beta-adrenergic receptor.

15. The method of claim 1, wherein the cardiovascular indication is associated with a condition selected from the group consisting of coronary artery occlusion, ischemic syndromes, cardiomyopathy, arrhythmia or dysrhythmia, infections, and an inflammatory condition.

16. The method of claim 15, wherein the condition of coronary artery occlusion results from or is associated with one selected from the group consisting of lipid/cholesterol deposition, macrophage/inflammatory cell recruitment, plaque rupture, thrombosis, platelet deposition, and neointimal proliferation.

17. The method of claim 15, wherein the condition of ischemic syndrome results from or is associated with one selected from the group consisting of myocardial infarction, stable angina, unstable angina, coronary artery restenosis and reperfusion injury.

18. The method of claim 15, wherein the condition of cardiomyopathy results or associated with from one selected from the group consisting of an ischemic syndrome, a cardiotoxin, an infection, hypertension, a metabolic disease, radiation, a neuromuscular disease, an infiltrative disease, trauma, and an idiopathic cause.

19. The method of claim 18, wherein the cardiotoxin is adriamycin.

20. The method of claim 18, wherein the infiltrative disease is one selected from the group consisting of sarcoidosis, hemochromatosis, amyloidosis, Fabry's disease, and Hurler's syndrome.

21. The method of claim 18, wherein the metabolic disease is one selected from the group consisting of uremia, beriberi, and glycogen storage disease.

22. The method of claim 15, wherein the condition of arrhythmia or dysrhythmia results from or is associated with one selected from the group consisting of ischemic syndrome, a cardiotoxin, adriamycin, an infection, hypertension, a metabolic disease, radiation, a neuromuscular disease, an infiltrative disease, trauma, and an idiopathic cause.
23. The method of claim 18, wherein the infection is caused by at least one selected from the group consisting of a bacterium, a virus, a fungus, and a parasite.
24. The method of claim 15, wherein the inflammatory condition is associated with at least one selected from the group consisting of myocarditis, pericarditis, endocarditis, immune cardiac rejection, and an inflammatory conditions resulting from one of idiopathic, autoimmune, and connective tissue diseases.
25. The method of claim 1, wherein the first therapeutic agent is an inhibitor.
26. The method of claim 25, comprising an inhibitor selected from the group consisting of an inhibitor of lipid or cholesterol synthesis or deposition, an inhibitor of macrophage or inflammatory cell recruitment or activation, a microtubule inhibitor, an anti-inflammatory agent, an anti-thrombotic agent, an anti-platelet agent, and an inhibitor of neointimal proliferation.
27. The method of claim 25, comprising an inhibitor of NF κ B.
28. The method of claim 27, comprising an inhibitor of NF κ B inhibitor selected from the group consisting of I κ B, PDTC, NAC metal chelators and anti-oxidants.
29. The method of claim 15, comprising a first therapeutic agent selected from the group consisting of an anti-apoptotic agent, a thrombolytic agent, a pro-angiogenic agent, a complement blocker, an inhibitor of reperfusion injury, a calcium channel blocker, a beta-blocker, an afterload reducer, a preload reducer, an anti-

thrombotic agent, an anti-platelet agent, anti-proliferative agent, an inhibitor of reactive oxygen metabolites, an anti-angiogenic agent, a biologically active fragment thereof, and a chimera thereof.

30. The method of claim 29, comprising an anti-proliferative agent selected from the group consisting of a ribozyme, an antisense oligonucleotide, an antibody, an inhibitor against one of c-myc, ras, raf, PI3 kinase, and cyclin, a suicide protein, a suicide gene, a proapoptotic protein, and a proapoptotic gene.

31. The method of claim 30, comprising an inhibitor selected from the group consisting of a protein, a peptide, an oligopeptide or a small molecule.

32. The method of claim 30, comprising the suicide protein Herpes thymidine kinase.

33. The method of claim 30, comprising suicide gene comprising a polynucleotide encoding Herpes thymidine kinase.

34. The method of claim 30, comprising a proapoptotic protein selected from the group consisting of fas, fab, and interleukin 1b converting enzyme.

35. The method of claim 30, comprising a proapoptotic gene comprises a polynucleotide encoding one selected from the group consisting of fas, fab and interleukin 1b converting enzyme.

36. The method of claim 11, comprising an anti-angiogenic agent selected from the group consisting of platelet factor 4, thrombospondin, a tissue inhibitor of a metalloproteinase, angiostatin, TFG- β , interferon- α , a proliferin-related protein, a biologically active fragment thereof, and a chimera thereof.

37. The method of claim 11, comprising an anti-tumor agent selected from the group consisting of a chemotherapeutic agent, a radiation sensitizer, and a radioactive implant.
38. The method of claim 11, comprising an antibiotic selected from the group consisting of an anti-viral agent, anti-trypanosomal agent, anti-bacterial agent, and an anti-fungal agent.
39. The method of claim 11, comprising an immunomodulating agent selected from the group consisting of a cytotoxic agent, a steroid, cyclosporin, and a complement inhibitor.
40. The method of claim 39, comprising a complement inhibitor selected from the group consisting of DAF, CAB-2, a biologically active fragment thereof, and a chimera thereof.
41. The method of claim 11, comprising an antiinflammatory agent selected from the group consisting of a steroid, a non-steroidal antiinflammatory agent, cyclosporin, a chemotherapeutic agent, and a complement inhibitor.
42. The method of claim 11, comprising an anti-arrhythmic agent selected from the group consisting of adenosine, quinidine, propranolol, digoxin, lidocaine, bretylium, amiodarone, and verapamil.
43. The method of claim 11, comprising an anti-hypertensive agent selected from the group consisting of hydralazine, propranolol, atrial natriuretic peptide, and an endothelin antagonist.
44. The method of claim 1, comprising a polynucleotide encoding a polypeptide selected from the group consisting of anti-apoptotic agent, a thrombolytic agent, a pro-angiogenic agent, an anti-arrhythmic agent, a contractility improving agent, a complement blocker, an inhibitor of reperfusion injury, an calcium channel blocker, a

beta-blocker, an afterload reducer, a preload reducer, an anti-thrombotic agent, an anti-platelet agent, anti-proliferative agent, an anti-inflammatory agent, an immunomodulating agent, an immunosuppressive agent, an inhibitor of reactive oxygen metabolites, an anti-angiogenic agent, a vasoactive agent, a cardioprotective agent, an iron-chelating agent, an anti-hypertensive agent, an anti-integrin agent, a pro-apoptotic agent, an anti-viral agent, an anti-parasitic agent, a free radical scavenger, a protein that may be deficient or downregulated during the development of cardiomyopathy, a biologically active fragment thereof, and a chimera thereof.

45. The method of claim 44, wherein the protein that may be deficient or downregulated during development of cardio-myopathy is troponin C or beta-adrenergic receptor.

46. The method of claim 1, comprising a polynucleotide encoding a polypeptide selected from the group consisting of an inhibitor of interleukin 1β converting enzyme, tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), urokinase, streptokinase, an inhibitor of $\alpha 2$ plasmin inhibitor, an inhibitor of plasminogen activator inhibitor-1 (PAI-1), basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), vascular endothelial cell growth factor (VEGF), angiogenin, transforming growth factor α (TGF- α), transforming growth factor β (TGF- β), tumor necrosis factor- α (TNF- α), platelet derived growth factor (PDGF), placental growth factor (PGF), hepatocyte growth factor, proliferin, decay accelerating factor, CAB-2, tissue factor pathway inhibitor (TFPI), heparin, hirudin, protein C, protein S, anti-thrombin III, tick anti-coagulant peptide (TAP), anti-stasin, glycoprotein IIb/IIIa antagonist, antibodies, Herpes thymidine kinase, fas, fap, platelet factor 4, thrombospondin, a tissue inhibitor of a metalloproteinase, prolactin, bFGF soluble receptor, a proliferin-related protein, myocyte growth factor, superoxide dismutase (SOD), troponin C, beta-adrenergic receptor, insulin-like growth factor I (IGF-I) a biologically active fragment thereof, and a chimera thereof.

47. The method of claim 1, wherein administering intrapericardially comprises delivery into the pericardial space by an administration selected from the

group consisting of injection, catheterization, laser-created perfusion channels, cannulization, a particle gun, and pump.

48. The method of claim 1 further comprising step (c) enhancing access of the first therapeutic agent to myocardial tissue prior to administering the pharmaceutical composition.

49. The method of claim 48, wherein enhancing access comprises a step selected from the group consisting of increasing penetration of myocardial tissue by the agent, and creating perfusion channels.

50. The method of claim 49, wherein increasing penetration comprises administering intrapericardially proangiogenic factor to increase vascularization of myocardial tissue.

51. The method of claim 49, wherein increasing penetration comprises using a formulation that increases tissue penetration of the therapeutic agent.

52. The method of claim 4, wherein the liposome comprises Depofoam®.

53. The method of claim 4, wherein the gel comprises Focalgel®.

54. The method of claim 9, wherein the liposome comprises Depofoam®.

55. The method of claim 9, wherein the gel comprises Focalgel®.

56. A kit for intrapericardial delivery of a pharmaceutical composition for treatment or prevention of a cardiovascular indication comprising a catheter for intrapericardial delivery and the pharmaceutical composition that comprises a polynucleotide.

57. The kit of claim 56, comprising a polynucleotide encoding a polypeptide selected from the group of an anti-apoptotic agent, a thrombolytic agent, a pro-angiogenic agent, an anti-arrhythmic agent, a contractility improving agent, a complement blocker, an inhibitor of reperfusion injury, an calcium channel blocker, a beta-blocker, an afterload reducer, a preload reducer, an anti-thrombotic agent, an anti-platelet agent, anti-proliferative agent, an anti-inflammatory agent, an immunomodulating agent, an immunosuppressive agent, an inhibitor of reactive oxygen metabolites, an anti-angiogenic agent, a vasoactive agent, a cardioprotective agent, an iron-chelating agent, an anti-hypertensive agent, an anti-integrin agent, a pro-apoptotic agent, an anti-viral agent, an anti-parasitic agent, a free radical scavenger, a protein that may be deficient or downregulated during the development of cardiomyopathy, a biologically active fragment thereof, and a chimera thereof.

58. The kit of claim 56, comprising a polynucleotide encoding a polypeptide selected from the group consisting of an inhibitor of interleukin 1β converting enzyme, tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), urokinase, streptokinase, an inhibitor of $\alpha 2$ plasmin inhibitor, an inhibitor of plasminogen activator inhibitor-1 (PAI-1), basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), vascular endothelial cell growth factor (VEGF), angiogenin, transforming growth factor α (TGF- α), transforming growth factor β (TGF- β), tumor necrosis factor- α (TNF- α), platelet derived growth factor (PDGF), placental growth factor (PGF), hepatocyte growth factor, proliferin, decay accelerating factor, CAB-2, tissue factor pathway inhibitor (TFPI), heparin, hirudin, protein C, protein S, anti-thrombin III, tick anti-coagulant peptide (TAP), anti-stasin, glycoprotein IIb/IIIa antagonist antibodies, Herpes thymidine kinase, fas, fap, platelet factor 4, thrombospondin, a tissue inhibitor of a metalloproteinase, prolactin, bFGF soluble receptor, a proliferin-related protein, myocyte growth factor, superoxide dismutase (SOD), troponin C, beta-adrenergic receptor, insulin-like growth factor I (IGF-I) a biologically active fragment thereof, and a chimera thereof.

59. The kit of claim 56, further comprising a drug.

60. The kit of claim 59, comprising a drug selected from the group consisting of an anti-apoptotic agent, a thrombolytic agent, a pro-angiogenic agent, an anti-arrhythmic agent, a contractility improving agent, a complement blocker, an inhibitor of reperfusion injury, a calcium channel blocker, a beta-blocker, an afterload reducer, a preload reducer, an anti-thrombotic agent, an anti-platelet agent, anti-proliferative agent, an anti-inflammatory agent, an immunomodulating agent, an immunosuppressive agent, an inhibitor of reactive oxygen metabolites, an anti-angiogenic agent, a myocyte growth factor, a vasoactive agent, a cardioprotective agent, an iron-chelating agent, an anti-hypertensive agent, an anti-integrin agent, a pro-apoptotic agent, an anti-viral agent, an anti-parasitic agent, a free radical scavenger, and a protein that may be deficient or downregulated during development of cardiomyopathy, and a biologically active derivative thereof.

61. A pharmaceutical composition for intrapericardial delivery for treatment of a cardiovascular indication comprising a polynucleotide.

62. The pharmaceutical composition of claim 61, further comprising a drug.

63. The pharmaceutical composition of claim 61, comprising a polynucleotide encoding a polypeptide selected from the group of anti-apoptotic agent, a thrombolytic agent, a pro-angiogenic agent, an anti-arrhythmic agent, a contractility improving agent, a complement blocker, an inhibitor of reperfusion injury, an calcium channel blocker, a beta-blocker, an afterload reducer, a preload reducer, an anti-thrombotic agent, an anti-platelet agent, anti-proliferative agent, an anti-inflammatory agent, an immunomodulating agent, an immunosuppressive agent, an inhibitor of reactive oxygen metabolites, an anti-angiogenic agent, a vasoactive agent, a cardioprotective agent, an iron-chelating agent, an anti-hypertensive agent, an anti-integrin agent, a pro-apoptotic agent, an anti-viral agent, an anti-parasitic agent, a free radical scavenger, a protein that may be deficient or downregulated during the development of cardiomyopathy, a biologically active fragment thereof, and a chimera thereof.

64. The pharmaceutical composition of claim 62, comprising a drug selected from the group consisting of an anti-apoptotic agent, a thrombolytic agent, a pro-angiogenic agent, an anti-arrhythmic agent, a contractility improving agent, a complement blocker, an inhibitor of reperfusion injury, a calcium channel blocker, a beta-blocker, an afterload reducer, a preload reducer, an anti-thrombotic agent, an anti-platelet agent, anti-proliferative agent, an anti-inflammatory agent, an immunomodulating agent, an immunosuppressive agent, an inhibitor of reactive oxygen metabolites, an anti-angiogenic agent, a myocyte growth factor, a vasoactive agent, a cardioprotective agent, an iron-chelating agent, an anti-hypertensive agent, an anti-integrin agent, a pro-apoptotic agent, an anti-viral agent, an anti-parasitic agent, a free radical scavenger, and a protein that may be deficient or downregulated during development of cardiomyopathy, and a biologically active derivative thereof.

65. The pharmaceutical composition of claim 61 wherein the polynucleotide comprises a viral vector.

66. The pharmaceutical composition of claim 65 comprising a viral vector selected from the group consisting of a retrovirus, an adenovirus, an adeno-associated virus, a herpes virus, an alpha virus, a semliki forest virus, and a sindbis virus.

67. The method of claim 1, further comprising administering to the patient a therapeutically effective amount of an anti-inflammatory agent to counteract an inflammatory response to administration of the first therapeutic agent, wherein the anti-inflammatory agent is administered before, contemporaneously with, or after administration of the first therapeutic agent.

68. The method of claim 3, further comprising administering to the patient a therapeutically effective amount of an anti-inflammatory agent to counteract an inflammatory response to administration of the first and second therapeutic agent, wherein the anti-inflammatory agent is administered before, contemporaneously with, or after administration of the first therapeutic agent.

69. The method of claim 7, wherein the viral vector comprises a herpes simplex thymidine kinase gene for inactivating the viral vector upon administration of gancyclovir.

70. The method of claim 1, further comprising administering a chaperone molecule before, contemporaneously with or after administration of the first therapeutic agent.

71. The method of claim 70, wherein the chaperone molecule is a heat shock protein.

72. The method of claim 71, wherein the heat shock protein is hsp70.

73. The method of claim 1, wherein the polynucleotide is linked to an inducible promoter.

74. The method of claim 1, wherein the polynucleotide is linked to a tissue specific promoter.

75. The method of claim 1, wherein the polynucleotide is flanked by nucleotide sequence suitable for integration into genome of myocytes.

76. The method of claim 11 comprising the proangiogenic factor is basic fibroblast growth factor (bFGF).

77. The method of claim 44 wherein the proangiogenic is factor basic fibroblast growth factor (bFGF).

78. The kit of claim 57 wherein the proangiogenic factor is basic fibroblast growth factor (bFGF).

79. The pharmaceutical composition of claim 63 wherein the proangiogenic factor is basic fibroblast growth factor (bFGF).

80. The method of claim 1 wherein the polynucleotide comprises L-aromatic amino acid decarboxylase.

81. The method of claim 80 further comprising administering a second therapeutic agent selected from the group consisting of L-Dopa, tyrosine, a polynucleotide encoding tyrosine hydroxylase, and a tyrosine hydroxylase polypeptide.

INTERNATIONAL SEARCH REPORT

Inter- national Application No
PCT/US 96/17311

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K9/00 A61K48/00 A61M29/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	<p>WO 96 39830 A (INDIANA UNIVERSITY FOUNDATION) 19 December 1996</p> <p>see claims 1-18 see page 10, line 1 - line 21 ---</p> <p>-/--</p>	<p>1,4,6-8, 12-26, 29,30, 44-47, 56-58, 61,62, 65,66, 77,78</p>

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- * "A" document defining the general state of the art which is not considered to be of particular relevance
- * "E" earlier document but published on or after the international filing date
- * "L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another claim or other special reason (as specified)
- * "O" document referring to an oral disclosure, use, exhibition or other means
- * "P" document published prior to the international filing date but later than the priority date claimed

* "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

* "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

* "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

* "Z" document member of the same patent family

Date of the actual completion of the international search

25 February 1997

Date of mailing of the international search report

14. 03. 97

Name and mailing address of the ISA

European Patent Office, P.B. 3118 Patentlaan 1
NL - 2210 HV Rijswijk
Tel. (+ 31-70) 340-2040, Telex 31 651 epo nl
Fax (+ 31-70) 340-3016

Authorized officer

Siatou, E

INTERNATIONAL SEARCH REPORT

Inter national Application No

PCT/US 96/17311

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	AMERICAN HEART JOURNAL, vol. 129, no. 5, May 1995, USA, pages 924-931, XP000618320 C. LANDAU ET AL: "Intrapericardial basic fibroblast growth factor induces myocardial angiogenesis in a rabbit model of chronic ischemia" see abstract ---	1-4, 6-12, 15-24, 29,30, 44-47, 56-66, 73-79
Y	WO 94 27612 A (BAYLOR COLLEGE OF MEDICINE) 8 December 1994 see claims 1-44 see page 33, line 14 - line 26 ---	1-4, 6-12, 15-24, 29,30, 44-47, 56-66, 73-79
A	GENE THERAPY, vol. 1, 1 January 1994, pages 51-58, XP000563621 E. BARR ET AL: "Efficient catheter-mediated gene transfer into the heart using replication-defective adenovirus" cited in the application see the whole document ---	1-81
A	CIRCULATION, vol. 91, no. 2, 15 January 1995, pages 541-548, XP000568740 E. G. NABEL: "Gene Therapy for Cardiovascular Disease" see the whole document ---	1-81
A	US 5 269 326 A (R. L. VERRIER) 14 December 1993 cited in the application see the whole document ---	1-81
A	WO 92 10221 A (MALLINCKRODT MEDICAL INC.) 25 June 1992 see the whole document & US 5 137 510 A cited in the application -----	1-81

INTERNATIONAL SEARCH REPORT

information on patent family members

later -all Application No

PCT/US 96/17311

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9639830 A	19-12-96	NONE	
WO 9427612 A	08-12-94	AU 7019494 A	20-12-94
US 5269326 A	14-12-93	AU 2916392 A	21-05-93
		CA 2122038 A	29-04-93
		EP 0746376 A	11-12-96
		WO 9307931 A	29-04-93
WO 9210221 A	25-06-92	US 5213570 A	25-05-93
		US 5137510 A	11-08-92
		AU 642840 B	28-10-93
		AU 9174191 A	08-07-92
		CA 2076009 A	15-06-92
		EP 0515676 A	02-12-92
		JP 5505131 T	05-08-93

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☒ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.